

ENZYMIC PROTEIN PHOSPHORYLATION

*a study of
membrane-bound protein kinases
in rat liver subfractions*

Marianne Sommarin



Lund 1983

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Lund, Sweden

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PUBLICATIONS

This thesis is based on the following papers, which are referred to by Roman numerals in the text.

- I Protein kinases of rat liver endoplasmic reticulum.
Solubilisation, partial characterisation and comparison with protein kinases of rat liver cytosol.
Sommarin, M. and Jergil, B.
Eur. J. Biochem. 88, 49-60 (1978)
- II Protein kinase activity and endogenous protein phosphorylation in rat liver plasma membranes.
Sommarin, M., Henriksson, T. and Jergil, B.
Acta Chem. Scand. B35, 5-12 (1981)
- III Cyclic AMP-dependent protein phosphorylation on the surface of rat hepatocytes.
Sommarin, M., Henriksson, T. and Jergil, B.
- IV Ca^{2+} - and phospholipid-dependent protein kinase in rat liver cytosol.
Sommarin, M. and Jergil, B.
Manuscript
- V Determination of Ca^{2+} - and phospholipid-dependent protein kinase in rat liver membranes.
Jergil, B. and Sommarin, M.
Biochim. Biophys. Acta, in press
- VI Microsomal Ca^{2+} - and phospholipid-dependent protein kinase -
- identification and in vitro binding studies.
Sommarin, M and Jergil, B.
Submitted for publication

INTRODUCTION

Protein phosphorylation is now recognized to be a major general mechanism by which intracellular events in mammalian tissues are controlled by external physiological stimuli. The plasma membrane usually acts as a transducer, receiving incoming signals and transforming them into internal signals which are responsible for regulating cellular activity.

The existence of covalently bound phosphorous in proteins has been known for more than a century, but its importance has only been realized since the discovery of enzyme regulation by phosphorylation-dephosphorylation reactions. This was mainly due to the work on glycogen metabolism in mammalian skeletal muscle performed by Krebs, Fischer and Larner. They discovered that the three enzymes glycogen phosphorylase (1), phosphorylase kinase (2), and glycogen synthetase (3) were regulated by changes in their state of phosphorylation.

Cyclic AMP was originally discovered during an investigation of the effects of adrenalin and glucagon on glycogen metabolism (4, 5). It was found that these two hormones stimulated liver glycogenolysis by increasing the concentration of cyclic AMP in the cell, which in turn stimulated the formation of an active phosphorylase. These findings were followed by other investigations demonstrating that cyclic AMP was present in almost every kind of cell and that the concentration of cyclic AMP was elevated by a number of hormones, and also caused many diverse effects in biological systems. The studies led to the second messenger hypothesis postulated by Sutherland (6). He suggested that cyclic AMP is a ubiquitous second messenger, the link between extracellular messengers and the control of intracellular events. The discovery of a cyclic AMP-dependent protein kinase in rabbit skeletal muscle (7) as an intracellular receptor for cyclic AMP, initiated an intense research effort which soon revealed that cyclic AMP-dependent protein kinases were ubiquitous and present in similar concentrations as cyclic AMP in all mammalian tissues (8). It became obvious that cyclic AMP-dependent protein kinases must have an essential role in mediating the diverse effects of cyclic AMP. A hypothesis was presented that the biological effect of a hormone is determined by whether its receptor is present on the plasma membrane surface of a target cell and by the physiological substrates for cyclic AMP-dependent protein kinase that are

present within the cell (9,10). This hypothesis is schematically outlined in fig. 1. If the appropriate hormone receptor is present on the plasma membrane surface, the hormone will bind to the receptor. The hormone-receptor complex activates adenylate cyclase present in the membrane, and cyclic AMP is produced from its precursor ATP inside the cell. Cyclic AMP binds to the regulatory subunit of the cyclic AMP-dependent protein kinase, causing dissociation of the inactive holoenzyme. The free and active catalytic subunits formed phosphorylate specific substrate proteins and a physiological response is achieved.

The importance of Ca^{2+} in cell function has been appreciated for a long time, but its molecular mechanism of action is only beginning to become unraveled. Work in the field of muscle physiology led to the conclusion that Ca^{2+} was the coupling factor or second messenger in stimulus-contraction coupling (11). Ca^{2+} was also found to be involved in stimulus-secretion coupling during the release of granules from a number of different cells (12,13). However, the importance of Ca^{2+} as a second messenger was not fully appreciated, perhaps in part because attention was diverted to the cyclic nucleotides. Renewed interest in Ca^{2+} -mediated cellular processes came from the suggestion that some of the different effects of Ca^{2+} are mediated through a homologous class of Ca^{2+} -receptors, in a way similar to the regulatory subunit of cyclic AMP-dependent protein kinase (14-16).

The major intracellular receptor for Ca^{2+} is calmodulin, a small, thermostable and acid-stable protein. Calmodulin is present in most mammalian tissues in concentrations ranging from 50-500 μg per g of tissue; the highest levels are found in brain and testis (15).

The Ca^{2+} -calmodulin complex acts in two ways, directly on an effector system such as an ATPase involved in transport, or indirectly on a regulatory system such as a protein kinase. Both fast (direct) and slow (indirect) responses are mediated by Ca^{2+} -calmodulin, in contrast to cyclic AMP, which is known to act only indirectly. The proposed mode of Ca^{2+} -calmodulin action, first established with the phosphodiesterase system (15-17), is depicted in fig. 1. The limiting factor in this scheme is the intracellular concentration of Ca^{2+} . Steady-state concentration of Ca^{2+} in the cytosol of mammalian cells ranges from 10^{-8} - 10^{-7} M. Stimulation of the cell may cause a transient increase of Ca^{2+} in the cytosol

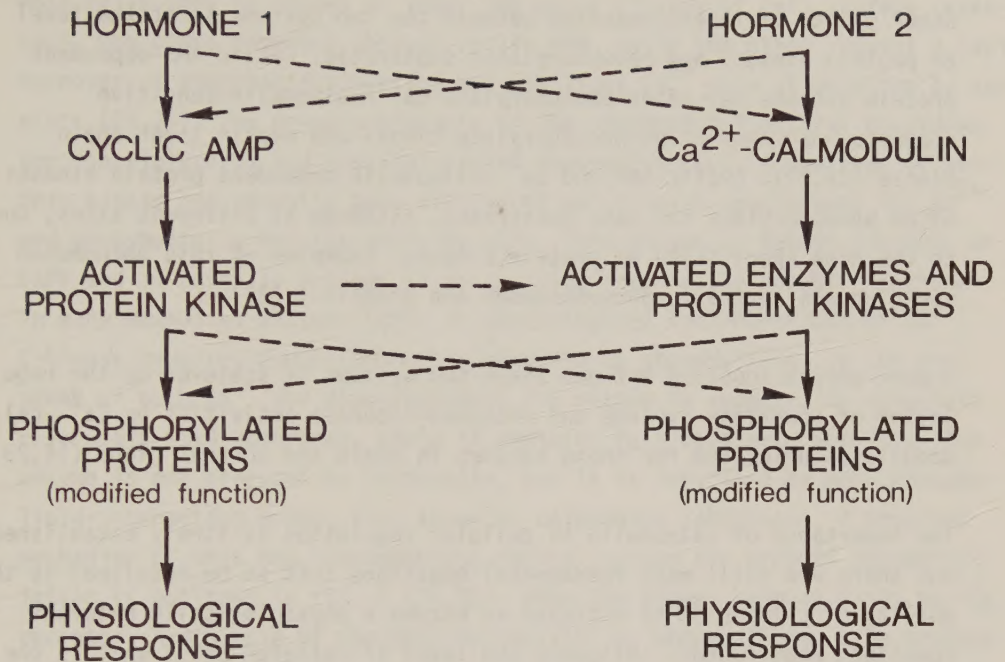


Fig. 1. Simplified scheme for the hormonal regulation of cellular functions through cyclic AMP and Ca^{2+} -calmodulin, and the relations between these two systems (14,22).

to 10^{-6} M or higher, and Ca^{2+} can then form an active complex with calmodulin (14,15). This complex in turn combines with an apoenzyme or effector protein (e.g. protein kinase) to elicit a biochemical response, leading to a physiological event. A major difference between the two regulatory systems (fig. 1), is that mammalian cells only contain one type of catalytic subunit of cyclic AMP-dependent protein kinase that phosphorylates a variety of intracellular substrates whereas Ca^{2+} -calmodulin affects a large number of calmodulin-dependent enzymes. In the case of protein kinases several are activated by Ca^{2+} -calmodulin, and each of them phosphorylates only a few substrates. Another important point, indicated by the broken lines between the two systems in fig. 1, is that these two regulatory systems are closely interrelated and may often be interchangeable in different tissues. Thus, the β -adrenergic stimulation of glycogenolysis in skeletal muscle is mediated by cyclic AMP, whereas the α -adrenergic effect in adult rat liver, at least in part, is mediated by Ca^{2+} (18,19).

Other points of interconnection between the two systems is at the level of protein kinases and phosphorylated substrates. Cyclic AMP-dependent protein kinases may often phosphorylate Ca^{2+} -calmodulin-sensitive enzymes. Two examples are phosphorylase kinase and myosin light chain kinase (20,21). Cyclic AMP and Ca^{2+} -calmodulin-dependent protein kinases often phosphorylate the same substrates, although at different sites, due to the high specificity of protein kinases. Examples of this phenomenon are glycogen synthase, phospholamban and protein-I (22).

A more direct coupling between these two systems is achieved by the regulation of adenylate cyclase and phosphodiesterase activities by Ca^{2+} -calmodulin as described for these enzymes in brain and adrenal gland (14,23,24).

The importance of calmodulin in cellular regulation is firmly established, but there are still many fundamental questions left to be resolved: is the ability of calmodulin to activate an enzyme a physiologically relevant reaction; what factors influence the level of cellular Ca^{2+} ; what is the molecular interrelation between Ca^{2+} , cyclic nucleotides and hormones, including prostaglandins? Before these questions and others are answered, the universal role of the Ca^{2+} -calmodulin regulatory system must be considered with care.

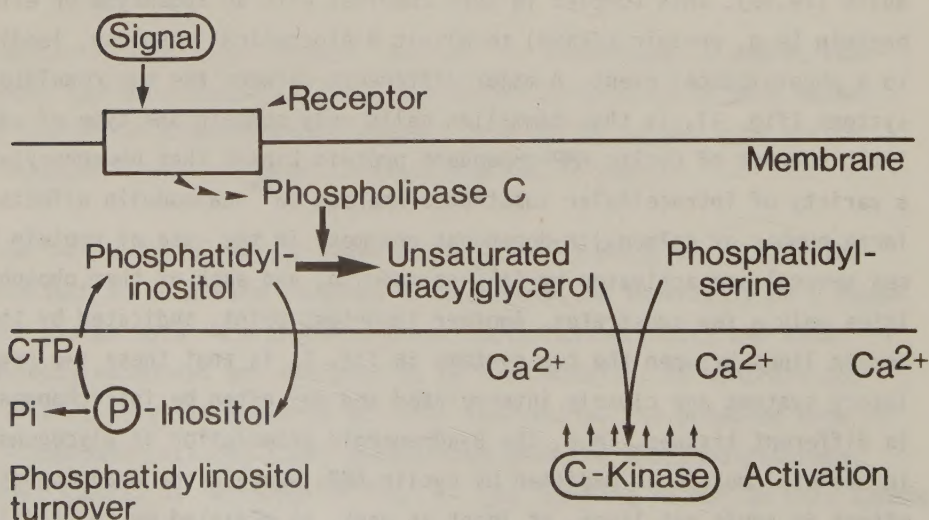


Fig. 2. A proposed mechanism of a transmembrane control system for protein phosphorylation (29).

Most cells seem to possess at least two major classes of cell surface receptors. One class operates through cyclic AMP, while the other induces a rapid turnover of phosphatidylinositol and mobilizes Ca^{2+} upon stimulation by agonists (25,26). The primary products of the phosphatidylinositol breakdown are diacylglycerol and inositol-cyclic-phosphates (26). A new type of protein kinase has recently been discovered which has a requirement for Ca^{2+} and phospholipids for its activity (27). This kinase is termed C-kinase or CaPK and is normally present in an inactive form in the soluble fraction in many mammalian tissues (28). At physiological concentrations of Ca^{2+} , C-kinase requires diacylglycerol in addition to phospholipid. In the presence of both Ca^{2+} and diacylglycerol the enzyme is supposed to associate reversibly with membranes, where it exhibits full catalytic activity. The enzyme is not affected by calmodulin, but it is inhibited by many phospholipid-interacting drugs, also known as calmodulin inhibitors. A proposed mechanism of this new transmembrane control system for protein phosphorylation is outlined in fig. 2 (29,30). When the proper hormone binds to its receptor, hydrolysis of phosphatidylinositol by phospholipase C to produce inositol phosphates and diacylglycerol is induced. The unsaturated diacylglycerol increases the affinity of C-kinase for Ca^{2+} and phospholipid, and serves as a second messenger for the selective activation of this C-kinase without a net increase in the Ca^{2+} concentration. The activated enzyme is supposed to be able to phosphorylate various regulatory enzymes and proteins.

This new type of protein kinase is suggested to play a crucial role in transmitting information from extracellular messenger to intracellular substrates, with diacylglycerol serving as a second messenger. However, further work is needed to test this hypothesis, since the events occurring after the stimulation of receptors is not known in molecular detail, the lipid-enzyme interaction is not understood, and physiological substrates for the protein kinase must be identified.

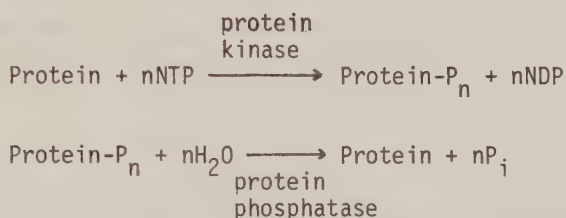
About 25 enzymes and more than 100 other proteins are now known to undergo phosphorylation-dephosphorylation reactions (31,32). The first three enzymes known to be regulated in this way (glycogen phosphorylase, phosphorylase kinase and glycogen synthase) are concerned with the regulation of glycogen metabolism in mammalian skeletal muscle. This system is still the best understood in molecular detail and is a model with which others are compared. The current knowledge of the control of glycogen metabolism has been reviewed in (8,22).

Protein phosphorylation is clearly the major general mechanism by which external physiological stimuli control intracellular events. There is an integrated network of regulatory pathways, mediated by phosphorylation-dephosphorylation and this allows different cellular functions to be controlled by the same protein kinases and protein phosphatases. Enzymes involved in biodegradative pathways are generally activated by phosphorylation, whereas enzymes involved in biosynthetic pathways are inactivated by phosphorylation. However, there is still much further information needed on the function of these systems in molecular detail to gain a full understanding of the large field of regulation of biological activity.

PROTEIN KINASES

Phosphate turnover

There is a relatively rapid turnover of protein-bound phosphate in many tissues from different animal species. This turnover is catalyzed by protein kinases and protein phosphatases through the following reaction cycle:



Protein kinases catalyze the transfer of the terminal phosphate group from a nucleosidetriphosphate (usually ATP, sometimes GTP) to a protein. Usually seryl or threonyl amino acid residues become phosphorylated in the substrate protein. Protein phosphatases catalyze the removal of protein-bound phosphate by hydrolysis. These enzymes have not been as thoroughly investigated as protein kinases, although those involved in glycogen metabolism have been isolated and characterized. Protein phosphatase-1 and -2A are active in the absence of divalent cations, while protein phosphatases-2B and -2C are completely dependent on Ca^{2+} and Mg^{2+} , respectively. Protein phosphatases exhibit a broad substrate specificity (with the exception of type -2B) and some of them can be regulated by specific inhibitors (22,31,33).

If a protein is to be regulated by phosphorylation-dephosphorylation reactions, certain signals must cause changes in the relative concentrations of the phosphorylated and dephosphorylated forms of the protein. This can be achieved by controlling either the protein kinase or protein phosphatase reaction, or both. The phosphorylation of an enzyme often causes a change in K_m for a substrate, K_a for an activator, or K_i for an inhibitor. Substrates, activators and inhibitors may thus amplify or suppress the effect of phosphorylation. Phosphorylation-dephosphorylation should therefore be regarded as a mechanism for switching an enzyme between two forms that respond differently to substrates and regulator molecules.

Classification of protein kinases.

Protein kinases have been classified into several subgroups with respect to their direct interaction with specific regulatory agents, effectors (31).

A. Protein kinases with known intracellular effectors.

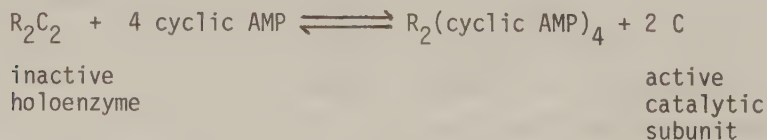
- 1) Cyclic AMP-dependent protein kinases
- 2) Cyclic GMP-dependent protein kinases (34,35)
- 3) Ca^{2+} -dependent protein kinases
- 4) Doublestranded RNA-dependent protein kinases (36)

B Protein kinases with no known intracellular effectors.

- 1) Protein kinases that phosphorylate seryl and threonyl residues
- 2) Protein kinases that phosphorylate tyrosyl residues (37,38,39)

Effects of cyclic AMP and Ca^{2+}

Cyclic AMP-dependent protein kinases are the most extensively studied kind of protein kinases. There exists two types of cyclic AMP-dependent protein kinases, type I and type II (40,41). It is generally accepted that both types are activated by the same overall reaction (35):



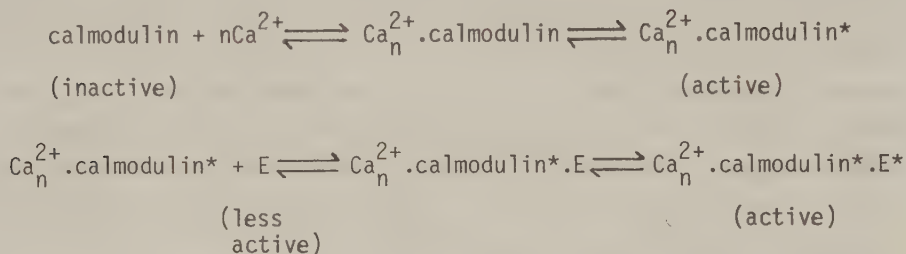
Cyclic AMP binds to the regulatory subunit of the inactive holoenzyme (R_2C_2), forming a ternary complex. The catalytic subunit dissociates from this complex and becomes free and active. The two types of cyclic AMP-dependent protein kinases have the same catalytic subunit but different regulatory subunits, which confers a substantial difference in physical and kinetic properties to these two isoenzymes. These differences include a difference in binding to ion-exchange columns, autophosphorylation of the type II (but not of the type I) regulatory subunit, differences in asso-

ciation-dissociation properties of the subunits, and different effects of MgATP, salt, histones and temperature (40-42). In comparison the cyclic GMP-dependent protein kinase consists of two apparently identical subunits that do not dissociate upon activation by cyclic GMP and contain separate catalytic and cyclic GMP-binding sites. This protein kinase can also undergo autophosphorylation (31). The cyclic AMP-dependent type I and type II protein kinases may also differ in their respective locations within the cell. In rat liver (I,II,43) and cardiac muscle (44,45) the type I enzyme has been found only in the cytosolic fraction, while the type II enzyme was also found in membrane fractions.

Tissues contain heatstable protein inhibitors of cyclic AMP-dependent protein kinases. The most well-studied one has been isolated from rabbit skeletal muscle (46,47). The inhibitor binds to the catalytic subunit and is competitive with respect to substrates for the protein kinase. The physiological function of the inhibitor is unknown, although the concentration of the inhibitor is influenced by different physiological conditions. Even in tissues where the protein kinase inhibitor is relatively abundant, only 20% of the cyclic AMP-dependent protein kinase activity can be blocked. Thus, the inhibitor might inhibit free catalytic subunits present under basal conditions, but it cannot be an important factor when the cell is activated. The protein kinase inhibitor is a useful tool for distinguishing the cyclic AMP-dependent protein kinases from other types of protein kinases (31). Some other types of protein kinase inhibitors have also been found (48,49).

There exist two classes of Ca^{2+} -dependent protein kinases, Ca^{2+} -calmodulin-dependent and Ca^{2+} - and phospholipid-dependent. Protein kinases known to be regulated by Ca^{2+} -calmodulin are myosin light chain kinase (15,50), phosphorylase kinase (15,51,52), glycogen synthase kinase (53), phospholamban kinase (54), tryptophan and tyrosine hydroxylase kinase (55,56), protein I kinase (57), and kinases responsible for the phosphorylation of endogenous proteins in membranes and soluble fractions from a variety of tissues whose identity and function is unknown (22). How Ca^{2+} -calmodulin activates these different protein kinases has not been conclusively determined. However there is not a calmodulin binding subunit analogous to the regulatory subunit of cyclic AMP-dependent protein kinases responsible for the multiple interactions. Only three of these Ca^{2+} -calmodulin dependent protein kinases (myosin light chain, phosphorylase and glycogen synthase kinases) have been purified and characterized (15).

The activation of Ca^{2+} -calmodulin-dependent enzymes is thought to consist of the following reactions (15-17):



Calmodulin binds Ca^{2+} and assumes a more helical conformation which is the active species. This active species then binds reversibly to the enzyme resulting in an active holoenzyme complex, which in the case of kinases is able to phosphorylate available protein substrates. However, the mechanism of activation by the Ca^{2+} -calmodulin cofactor of this heterogenous class of protein kinases, as well as a description of their characteristics and common features, needs to be further investigated.

The Ca^{2+} - and phospholipid-dependent protein kinase, called C-kinase or CaPK, has been shown to occur ubiquitously in various tissues and organs (28). The enzyme shows no apparent tissue and species specificity in physical, kinetic and catalytic properties. In many tissues the activity of this enzyme far exceeds that of cyclic AMP-dependent protein kinase, and the enzyme is most abundant in brain and platelets. The enzyme has recently been extensively purified from the soluble fraction of bovine heart (58,59) and rat brain (60). The C-kinase is a monomeric protein with two distinctly different domains, a hydrophobic that may bind to membranes, and a hydrophilic one that contains the catalytic site (30). The C-kinase exists both as a soluble and membrane-bound enzyme (30,IV,VI). The mechanism by which Ca^{2+} and phospholipid activate the enzyme remains to be elucidated.

Substrate specificities

A number of substrates for protein kinases exist and can be divided into two major groups, namely enzymic and non-enzymic proteins. The function of covalently bound phosphate in enzymes may be that of regulating the enzyme activity, or alternatively, the phosphate may have a role in maintaining the structure of the enzyme without affecting its activity(61). Another kind of

phosphorylation is when the active site becomes transiently phosphorylated during the course of a reaction cycle which proceeds through the formation of a phosphorylated enzyme intermediate. This kind of phosphorylation is not catalyzed by protein kinases, and will not be dealt with here. The function of covalently bound phosphate in non-enzymic proteins can be that certain phosphoproteins may serve as stores of phosphate or bound metal ions (61). The phosphorylation of other proteins may alter their functional and structural properties (61). Although the hormonal regulation of enzyme activities and the control of intermediary metabolism through phosphorylation-dephosphorylation reactions have received the greatest attention, perhaps most hormonal effects are concerned with processes where the phosphorylatable proteins are non-enzyme proteins.

Protein kinases phosphorylate a number of different substrates. However, it is important to realize that they are highly specific enzymes, since only a few sites on the proteins are phosphorylated at significant rates. Only one or a few seryl or threonyl residues are phosphorylated in a physiological substrate, even though many such residues are present in a protein molecule. It has been shown that the primary structure plays a major role in determining the target site for cyclic AMP-dependent protein kinases. The minimum information needed for yielding a significant rate of phosphorylation is provided by an amino acid sequence of the general type Arg-Arg-X-Ser(P) or Lys-Arg-X-X-Ser(P) (31). The presence of two adjacent basic amino acids on the N-terminal side of the phosphorylatable seryl or threonyl residue is thus essential for substrate recognition. However, the three-dimensional structure of the potential target may also be of importance (31,62). The substrate specificities of other kinds of protein kinases than the cyclic AMP-dependent ones are also likely to be determined by amino acid residues present in the vicinity of the site to be phosphorylated and the tertiary structure of the protein.

Different protein kinases are often able to phosphorylate the same substrates and this has been used in characterization studies. Histones, prothamine, casein and phosvitin are examples of such common substrates. When different protein kinases recognize the same substrate they usually phosphorylate different sites. This phenomenon is called multisite phosphorylation, and provides a simple mechanism whereby proteins are regulated by several physiological stimuli. Phosphorylation at one site may stimulate or inhibit the effects of phosphorylation at other sites, or alter the rate of phosphorylation or dephosphorylation.

Originally Krebs proposed a number of criteria that should be satisfied in order for a protein to serve as a physiological substrate for cyclic AMP-dependent protein kinase in vivo (63). These criteria have been redefined to include phosphorylation by enzymes other than the cyclic AMP-dependent protein kinases (31):

1. Demonstration in vitro that the enzyme can be phosphorylated stoichiometrically at a significant rate in a reaction(s) catalyzed by an appropriate protein kinase(s) and dephosphorylated by a phosphoprotein phosphatase(s).
2. Demonstration that functional properties of the enzyme undergo meaningful changes that correlate with the degree of phosphorylation.
3. Demonstration that the enzyme can be phosphorylated and dephosphorylated in vivo or in an intact cell system with accompanying functional changes.
4. Correlation of cellular levels of protein kinase and/or phosphoprotein phosphatase effectors and the extent of phosphorylation of the enzyme.

The same set of criteria can be applied also to enzyme phosphorylated at multiple sites, but then it is necessary to examine the significance of each phosphorylation event separately.

About 25 enzymes are now known to be regulated by phosphorylation-dephosphorylation reactions, but so far only phosphorylase kinase, glycogen synthase and pyruvate kinase meet these four criteria and can be said to serve as physiological substrates for cyclic AMP-dependent protein kinase in vivo (22).

PROTEIN PHOSPHORYLATION IN RAT LIVER ENDOPLASMIC RETICULUM

General aspects of rat liver endoplasmic reticulum

The adult rat liver contains two major types of cells, parenchymal cells (hepatocytes) and nonparenchymal or sinusoidal lining cells (Kupffer cells) in approximately equal numbers (64). The endoplasmic reticulum prepared from rat liver originates chiefly from hepatocytes since the other cell types are much smaller and contain a limited amount of endoplasmic reticulum. As an example the activities of the microsomal enzymes glucose-6-phosphatase and benzo(a)pyrene hydroxylase is negligible in isolated sinusoidal cells (65-67). Furthermore, these smaller cells are not disrupted by normal homogenization procedures (68).

The endoplasmic reticulum of hepatocytes is a complex membrane network extending from the nuclear envelope throughout the cytoplasm to the cell membrane. This membrane network is composed of vesicles, tubules and lamellae (69,70). Several enzymes and enzyme systems carrying out a great variety of important biochemical reactions are localized to the endoplasmic reticulum. These include enzymes involved in the synthesis and transport of a number of proteins, including glycoproteins and lipoproteins, enzymes involved in the synthesis of cholesterol, phospholipids and triglycerides, in the metabolism of xenobiotics, and enzymes participating in glycogen breakdown. The endoplasmic reticulum responds to changes in the physiological state of the animal, such as exposure to xenobiotics, by increasing its volume and surface area together with induction of drug metabolizing enzymes (71,72). The adaptability and functional importance of the endoplasmic reticulum has made it an object of numerous investigations.

Morphometric studies reveal that the endoplasmic reticulum of hepatocytes occupies approximately 15 % of the total cell volume (70). The surface has been estimated to be around 11 m^2 per ml of liver tissue, i.e. about 38 and 9 times the surface areas of the plasma membrane and the outer mitochondrial membrane, respectively (70). Approximately 60% of the cytoplasmic surface of the endoplasmic reticulum is covered with ribosomes, and is called the rough endoplasmic reticulum. This is usually arranged in parallel arrays of broad flattened sacs, called cisternae. The remaining area, the smooth endoplasmic reticulum, is often found close to the Golgi region, and consists of widely dispersed tubules and vesicles sometimes associated with glycogen deposits (69,70).

Biochemical studies show that the endoplasmic reticulum contains around 60% of the total RNA, 50% of the phospholipid and 20% of the protein of the rat hepatocyte (73). The endoplasmic reticulum membrane is composed of 70% protein and 30% lipid (of which 85% is phospholipid). The phospholipids and most peripheral and integral proteins are asymmetrically distributed in the transverse plane of the membrane (73).

Preparation of endoplasmic reticulum

The liver endoplasmic reticulum is extensively disrupted even by gentle homogenization procedures in contrast to other organelles such as mitochondria and lysosomes. This breakage seems to involve an active "pinching off" process (74). Upon homogenization the endoplasmic reticulum breaks transversely into a vesicular and a lamellar fraction, and subfractionation might reveal heterogeneities in the lateral plane of this organelle. The vesicular material is recovered by differential centrifugation in the microsomal fraction (100 000 xg pellet) while the lamellae sediments at low centrifugal forces (640 xg). This rapidly sedimenting endoplasmic reticulum is structurally associated with mitochondria (75). The microsomal and the rapidly sedimenting endoplasmic reticulum fractions can be further subfractionated by discontinuous sucrose gradient centrifugation into smooth and rough microsomes and smooth and rough rapidly sedimenting endoplasmic reticulum, respectively. Sometimes the rough subfractions are further fractionated into light and heavy rough fractions (76).

Protein kinases of rat liver endoplasmic reticulum (I)

Enzymic phosphorylation of proteins occurs in all the major subcellular fractions of rat liver, i.e. cytosol (77-79), mitochondria (43,80), Golgi membranes (81), plasma membranes (II,81,82), ribosomes (83,84), and endoplasmic reticulum (81,85-88). Several studies have shown that a number of rat liver microsomal polypeptides can be phosphorylated by endogenous protein kinases (81,85-88), but no attempt to isolate and characterize the membrane-bound protein kinases involved in this phosphorylation had been done until the investigation presented in I was performed.

Approximately 70% of the total protein kinase activity (using protamine as a substrate) was found in the soluble portion of the cell, whereas the remaining kinase activity was distributed between nuclei, mitochondria,

rapidly sedimenting endoplasmic reticulum and microsomes. The protein kinase activity of microsomes was around 10% of the total activity in liver cells. The lower protein kinase content of 5% in microsomes reported by Chen and Walsh (77) was due to their use of histones as substrate. A cyclic AMP-independent protein kinase, which does not readily phosphorylate histones makes up a substantial part of the microsomal kinase activity, and this enzyme was therefore not detected by Chen and Walsh. We found a two-fold enhancement of the kinase activity by Triton X-100 in microsomes and rapidly sedimenting endoplasmic reticulum, thus revealing the presence of a hidden activity in the membrane.

The microsomal protein kinase activity is tightly bound to the membrane. Harsh treatment of the microsomes with 1.5 M KCl and sonication released only a part of this enzyme activity. The kinase could be extracted by the addition of detergent. A slightly higher concentration of Triton X-100 was needed to solubilize the protein kinase than to solubilize several microsomal marker enzymes.

Solubilization of the microsomal protein kinase activity followed by DEAE-cellulose chromatography revealed three protein kinase peaks designated M I, M II and M III. Protein kinase fraction M III was dependent on cyclic AMP for maximum activity. DEAE-cellulose chromatography of rat liver cytosol under the same conditions as for the microsomal extract yielded five protein kinase peaks designated C Ia, C Ib, C IIa, C IIb and C III. Fractions C IIa and C III were activated by cyclic AMP, although to different extents. The microsomal and cytosolic protein kinase fractions were partially characterized and compared as to their isoelectric points, activation by cyclic AMP and substrate specificities. The results indicated the existence of both cyclic AMP-dependent and -independent protein kinases in cytosol as well as in microsomes. Fractions M II and C IIb were cyclic AMP-independent enzymes, and have later been identified as the Ca^{2+} - and phospholipid-dependent protein kinase (IV,VI). Fraction C IIa was the type I and C III the type II cyclic AMP-dependent protein kinase, while C Ia and C Ib represented the free catalytic subunit of these enzymes. The difference between C Ia and C Ib has not been examined. The microsomal M I and M III corresponded to the cytosolic fractions C Ia and C III, respectively. Thus, the type I enzyme is found only in the cytosol, while the type II enzyme together with the Ca^{2+} - and phospholipid-dependent enzyme exists both in a soluble and a membrane-bound form. The two cyclic AMP-dependent protein kina-

ses have similar catalytic but different regulatory subunits, and it is likely that the latter determines whether the enzyme would bind to membranes. Whether the small amount of free catalytic subunit present in the microsomal membrane extract represents subunits free in the membrane in vivo, or merely is due to the partial dissociation of the type II enzyme during the extraction procedure is not known. Since this report was published microsomal protein kinases were analyzed in a similar way by Tan et al. (89). No additional results that extended the knowledge of the molecular properties and physiological function of the microsomal protein kinases were presented. Uno et al. (90) resolved microsomal protein kinases from bovine liver into two cyclic AMP-dependent protein kinase fractions. Their first eluting peak probably represented the type I cyclic AMP-dependent protein kinase, that we have found to exist in the cytosol. However, Uno et al. did not wash their membranes with salt. We have found that a small fraction of the type I enzyme has the same loose association with microsomes as cytosolic marker enzymes. It is therefore likely, that their first peak merely represented a contamination from the cytosol. Their second peak probably corresponded to the type II cyclic AMP-dependent protein kinase. Uno et al. used histones as substrate when analyzing their chromatogram, and since histones are poor substrates for the Ca^{2+} - and phospholipid-dependent protein kinase, unless Ca^{2+} and phospholipid are also present, they could not detect this protein kinase.

The ability of the different protein kinase fractions to bind to smooth microsomes was studied. Enzyme fraction C IIb bound most efficiently followed by C III, while fractions C Ia and C IIa did not bind appreciably. The binding of the microsomal enzymes M II and M III was rather low, probably due to disturbance of the enzyme-membrane interaction by trace amounts of Triton X-100 still present in the enzyme fractions. After identification of the cyclic AMP-independent enzyme (C IIb) as the Ca^{2+} - and phospholipid-dependent protein kinase we have reexamined its binding to microsomal membranes in order to establish factors involved in this binding (VI). Ca^{2+} was found to be necessary for binding presupposed the microsomes were prepared in buffer containing EGTA to remove Ca^{2+} tightly associated with the membrane.

The transverse distribution of protein kinases M II and M III in the microsomal membrane was investigated by their sensitivity to trypsin treatment. Since trypsin is not able to penetrate the microsomal membrane, only proteins of the outer surface of intact vesicles are susceptible to proteolytic attack (91,92). After trypsinization only 20% of the protein kinase activity originally present remained when analyzed without detergent (using protamine as substrate), while 50% was left when detergent was included in the assay mixture. 70% of the protein kinase fraction M II was lost compared to only 20% of the M I and M III activity as judged by DEAE-cellulose chromatography before and after trypsin treatment of microsomes. It is therefore likely that M II is located on the outer surface of the microsomal vesicle. M I and M III seemed to be hidden in the membrane, and a luminal location was suggested by later experiments using a synthetic peptide for cyclic AMP-dependent protein kinases. Very little protein kinase activity was detected in microsomes unless Triton X-100 was added. Then a 10-fold stimulation of the activity was obtained as compared to 2-3-fold when protamine was used as substrate. Since the latent microsomal protein kinase activity was not extracted by treatment with low amounts of deoxycholate or Triton X-100 known to release soluble luminal proteins, it is implied that this enzyme is attached to the luminal surface of the microsomal membrane.

Smooth and rough microsomes both contain the type II enzyme and the catalytic subunit of the cyclic AMP-dependent protein kinase together with the Ca^{2+} - and phospholipid-dependent protein kinase. When smooth and rough microsomes were phosphorylated endogenously and then subjected to SDS-polyacrylamide gel electrophoresis, the resulting phosphoprotein patterns showed some differences (81,87). Cyclic AMP did not enhance the phosphorylation of any of these components, but since the type II enzyme and the catalytic subunit of the cyclic AMP-dependent protein kinases presumably are present on the luminal side, it is uncertain whether they would participate in this phosphorylation. Furthermore, when the microsomal subfractions were phosphorylated endogenously in the presence of detergent additional phosphorylated components appeared, suggesting that the intact membrane imposed some restrictions on the phosphorylation. The transverse location in the membrane of these additional components is not known, but they may be located on the luminal side or are otherwise buried in the membrane. The identity and function of the microsomal phosphoproteins remain unclear, but it can be assumed that the phosphorylation represents regulatory modi-

fications. The different transverse location of the protein kinases probably reflects different physiological functions in the microsomal membrane. 3-Hydroxy-3-methylglutaryl coenzyme A reductase, a key enzyme in cholesterol synthesis, is present in the endoplasmic reticulum and is regulated by reversible phosphorylation (93,94). Another example of a microsomal phosphoprotein is acetyl coenzyme A carboxylase, an enzyme involved in fatty acid synthesis in both the soluble and microsomal part of the cell (95).

PROTEIN PHOSPHORYLATION IN RAT LIVER PLASMA MEMBRANES

General aspects of rat liver plasma membranes.

The plasma membrane plays an important regulatory role in cellular metabolism and function. It is the primary site of the action of several hormones, thus enabling the cell to recognize and respond properly to external signals. The plasma membrane is involved in cell-to-cell adhesion and communication, and has also a number of transport functions.

The hepatocyte, an irregular or elongated polyhedron about 25-30 μm by 20-25 μm , is a highly polarized cell. The plasma membrane is laterally differentiated into three distinct regions which have different functions (96,97). One is the blood sinusoidal region, which has a large microvillar surface that is specialized for the exchange of metabolites with the blood and for binding of hormones. The bile canalicular region has a smaller microvillar surface area, that is enclosed by tight junctions. This region participates in bile secretion and in the excretion of catabolites and products detoxified in the hepatocyte. The blood sinusoidal and bile canalicular regions are separated by the contiguous or lateral surface area. The desmosomes, gap junctions and tight junctions of this area allow structural and functional interactions with neighbouring cells. An estimate of the relative surface areas of the three different regions shows that the blood sinusoidal region comprises 72%, while the contiguous and bile canalicular regions constitute 15 and 13%, respectively, of the total hepatocyte surface area (98). The plasma membrane surface area of hepatocytes is approximately 74% of the total for all liver cells (99). Studies on the distribution of a number of marker enzymes between the plasma membrane regions reveal that the enzymes are unevenly distributed on the hepatocyte surface membrane. For example, 5'-nucleotidase and alkaline phosphodiesterase are present at the highest specific activities relative to the homogenate in the bile canalicular fractions (100), while glucagon-activated adenylate cyclase may have a primarily blood sinusoidal plasma membrane location (100). Na^+ , K^+ -ATPase is found in both the contiguous and blood sinusoidal plasma membrane fractions (101,102). The plasma membrane proteins are asymmetrically distributed in the transverse plane (97,103). Plasma membranes contain the highest amounts of glycosylated proteins and lipids relative to other membranes. The liver plasma membrane is characterized by a high choleste-

rol/phospholipid ratio and a high sphingomyelin content (104). The transverse distribution of phospholipids is different in the three functional regions, although the differences are less pronounced than the differences observed in polypeptide and enzyme composition.

Preparation of plasma membranes

Preparation of plasma membrane fractions corresponding to each of the three different regions has been achieved (100). The original method for the preparation of rat liver plasma membranes was developed by Neville (105) and modified by Emmelot et al. (106). Today numerous preparation methods exist. Common to all of them is an initial gentle homogenization to disrupt the hepatocytes with low shear forces. The microvillar blood sinusoidal region of the plasma membrane then forms vesicles, predominantly of the right side out configuration (107,108). The low shear forces also release large strips of plasma membranes that are attached by tight junctions to relatively intact bile canaliculi. The plasma membrane fragments predominantly originating from the bile canaliculi and contiguous regions, together with small variable amounts of blood sinusoidal membranes, are sedimented at low speed centrifugation together with nuclei, rapidly sedimenting endoplasmic reticulum, heavy mitochondria, nonparenchymal cells and cell debris. The main portion of the blood sinusoidal vesicles needs higher centrifugal forces to be pelleted. These vesicles sediment with the microsomes at 100 000 xg. Plasma membranes sedimenting with both the nuclear and microsomal fractions are further purified by density-gradient centrifugation.

We prepare plasma membrane fractions according to the method described by Aronson and Touster (109). Plasma membranes obtained from the nuclear fraction, designated N_2 , consist of vesicles and some junctional complexes most likely corresponding to the bile canaliculi and lateral plasma membrane regions (110). Plasma membranes obtained from the microsomal fraction, designated P_2 , are derived from the blood sinusoidal region, and contain small vesicles with fibrous material and vesicles loaded with very-low-density lipoprotein particles (110). The latter indicates a substantial contamination by the Golgi apparatus (111). The relative specific activity of the Golgi marker enzyme N-acetylglucosamine galactosyltransferase compared to the tissue homogenate was 28 for the P_2 -fraction and 6 for the N_2 -fraction. Corresponding values for the plasma membrane marker

enzyme 5'-nucleotidase were 20 and 34, respectively. We have used the N_2 fraction for the characterization of protein kinases and phosphoprotein pattern, since this plasma membrane fraction at the time was the only one purified to a high degree. Corresponding plasma membrane preparations have been used by most other investigators of plasma membrane function. Recently, we have developed a rapid and convenient method to separate the contaminating Golgi material from the blood sinusoidal vesicles by using two-phase partitioning technique, thus obtaining a rather pure blood sinusoidal plasma membrane preparation (110). The relative specific activity of the Golgi marker enzyme was 3 for plasma membranes obtained by using the two-phase partitioning technique compared to 28 for the P_2 fraction obtained by sucrose-density centrifugation. Corresponding values for the plasma membrane marker enzyme were 26 and 20, respectively.

Protein kinases and phosphoproteins in isolated rat liver plasma membranes (II)

Phosphorylation of plasma membrane proteins occurs in a number of cell types, e.g. erythrocytes, nerve cells, fibroblasts, adipocytes and ovary cells (112-115). Comparatively little is known about protein kinases and phosphoproteins in rat liver plasma membranes. Incorporation of ^{32}P -phosphate into rat liver plasma membrane proteins was first shown by Blat and Harel (116,117), but no further characterization of the phosphorylated proteins was done. Schlatz and Marinetti (118) found that rat liver plasma membrane proteins could be phosphorylated by cytosolic protein kinases in a cyclic AMP-dependent manner, and recently Carstens and Weller (119) found endogenous cyclic AMP-dependent phosphorylation in this membrane fraction. However, no attempt to characterize the protein kinases and phosphoproteins involved was made by these investigators. Recently, Marchmont and Houslay claimed that peripheral low K_m cyclic AMP phosphodiesterase was regulated by insulin- and cyclic AMP-dependent phosphorylation (120,121).

In view of the scanty knowledge a characterization of protein kinases and the phosphoprotein pattern in rat liver plasma membranes was performed (II).

Plasma membranes require a much higher ATP/membrane protein ratio than other rat liver membranes in order to obtain optimum conditions for the incorporation of phosphate into both exogenous and endogenous substrates. This is due to ATPase present in the plasma membrane, and therefore the ATPase inhibitor sodium azide was included in the assay mixture for endo-

genous phosphorylation. The specific activity of the plasma membrane protein kinase was much higher than the activity found in microsomes (I) and mitochondria (43). Cyclic AMP did not significantly influence the phosphorylation of the endogenous or exogenous substrates used, although we found cyclic AMP binding material present in the membrane. When isolated hepatocytes were phosphorylated with [γ -³²P] ATP there was a marked stimulatory effect of added cyclic AMP (III). Furthermore, the phosphorylation of both endogenous and exogenous substrates in isolated plasma membranes was inhibited by the rabbit skeletal muscle protein kinase inhibitor. Other research groups have found either a slight stimulatory effect of cyclic AMP, although the activity could not be inhibited by beef heart protein kinase inhibitor (119), or a decrease in the total incorporation of [³²P]-phosphate into membrane protein when cyclic AMP was present (122-124). However, no attempt to further characterize protein kinases involved was made by these research groups (119,122-124). These results together with ours implicate the existence of cyclic AMP-dependent and -independent protein kinases in rat liver plasma membranes. No latency of protein kinase activity was seen by us and others (119) when the plasma membranes were treated with Triton X-100, in contrast to what we have found earlier in microsomes (I) and mitochondria (43). This suggests that the substrates were freely accessible to the membrane-bound protein kinase. If the vesicles are sealed, this indicates that the protein kinases are attached to one surface only. This is probably the outer surface, since it has been reported that vesicles formed during the preparation of plasma membranes are predominantly of the right side out configuration (107,108,125).

The major portion of the protein kinase activity of plasma membranes could not be extracted unless both high salt and detergent were present in the extraction medium, implicating that the protein kinases are tightly bound to the membrane. DEAE-cellulose chromatography of the plasma membrane extract revealed two protein kinase peaks, designated PM I and PM II. These were characterized by their substrate specificities, isoelectric points, and the effects of the rabbit skeletal muscle protein kinase inhibitor. The PM I fraction most likely represented the catalytic subunit of the cyclic AMP-dependent protein kinases, while the identity of PM II was less clear. The PM II fraction exhibited characteristics indicating that this fraction may consist of a mixture of cyclic AMP-independent and cyclic AMP-dependent type II protein kinase. Occasionally, a third plasma membrane protein kinase peak was observed, which eluted at the same KCl

concentration as C IIb from the cytosol (I) and M II from microsomes and mitochondria (I,43). The reason why the type II enzyme and the cyclic AMP-independent protein kinase sometimes are eluted as a complex and sometimes as isolated entities is not known. However, the high concentration of glycoproteins in the plasma membrane extract may favour aggregation of various components. We have found later that a cyclic AMP-dependent protein kinase is present on the outer surface of isolated hepatocytes (III). Furthermore, we also detected the Ca^{2+} - and phospholipid-dependent protein kinase in isolated plasma membranes (V). These results confirm that rat liver plasma membranes contain the catalytic subunit and the type II cyclic AMP-dependent protein kinase together with the Ca^{2+} - and phospholipid-dependent protein kinase.

The phosphoprotein pattern of plasma membranes was also analyzed. Only a few proteins became heavily phosphorylated upon incubation with $[\gamma^{32}\text{P}]\text{ATP}$, and most of these were tightly bound to the particulate material. No effect of cyclic AMP on the phosphoprotein pattern was seen. Contrarily, Marchmont and Houslay (120,121) have reported that cyclic AMP stimulates the phosphorylation of two integral proteins, and when added together with insulin three peripheral proteins. One of the latter proteins was the low K_m cyclic AMP phosphodiesterase. I have repeated their experiments with our plasma membrane preparation, but did not observe any effect of cyclic AMP alone, or in combination with insulin, on the phosphoprotein pattern. Others have also failed to demonstrate phosphorylation of any proteins that might correspond to these integral or peripheral membrane proteins (126). The discrepancy between the results obtained by Marchmont and Houslay (120,121) and by us may be due to their use of a rather impure plasma membrane preparation (with only half the relative specific activity of 5'-nucleotidase compared to our preparation) although both preparations were derived from the nuclear pellet. Our plasma membrane fraction contains a large proportion of bile canaliculi vesicles (110), and since hormone receptors are preferentially localized to the blood sinusoidal region, we would not expect to see appreciable hormonal effects in this membrane fraction. Thus, when hormonal effects are to be studied, the best plasma membrane fraction should be the one derived from the blood sinusoidal region, which is predominantly recovered in the microsomal fraction. However, this membrane fraction has not been used in most investigations on hormonal effects.

The identity of the phosphorylated proteins in plasma membrane and their functional importance remain to be established. Some phosphorylatable plasma membrane proteins that have been suggested to be regulated by reversible phosphorylation are the peripheral low K_m phosphodiesterase of rat liver (120,121), the Na^+K^+ - ATPase of rat kidney (127), histocompatibility antigens from lymphocytes (128) and the insulin receptor from rat liver (39). It is notable that the insulin receptor is phosphorylated at tyrosol residues. Membrane protein phosphorylation has been shown to mediate membrane permeability in neural and other tissues (128,129). Furthermore, the interaction of membrane proteins with the cytoskeleton might be regulated by phosphorylation-dephosphorylation; as an example, the phosphorylation of spectrin (a membrane-bound protein in erythrocytes) changes its interaction with actin in vitro (130).

Preparation of hepatocytes.

Dissociation of tissues into single cells involves both the dissolution of the extracellular matrix and the breakage of cell-to-cell contacts without breaking the cell membrane. As discussed earlier, the cells in a tissue are kept together by desmosomes, tight junctions and gap junctions. Extraction of intercellular Ca^{2+} irreversibly cleaves the desmosomes into hemidesmosomes, that move apart and become internalized (131). No satisfactory methods are known for the cleavage of tight or gap junctions, but the cells are apparently capable of being torn apart at these sites. The whole junction is left attached to the surface of one cell, whereas the plasma membrane is resealed at the junctional site of the other cell (132).

Hepatocytes were prepared according to the collagenase perfusion technique originally applied to rat liver by Berry and Friend (132) and further developed by Seglen (133). This technique is the best tissue dispersion method known today. The liver is perfused at 37 °C in two stages. First, extracellular Ca^{2+} is removed by extensive perfusion with Ca^{2+} -free buffer or EGTA in order to cleave the desmosomes. In the second stage the extracellular matrix is digested by 0.05% collagenase. To enhance the viability of the cells 0.1% hyaluronidase and 0.2% bovine serum albumin were included in the perfusion medium. The initial cell suspension obtained after collagenase treatment consists mostly of hepatocytes, some small nonparenchymal cells, capsule fragments, and subcellular debris. This suspension is preincubated for 30 min in a rotating waterbath at 37 °C in order to cause intact cells to round up and damaged cells to loose its contents and

become less dense. This facilitates the subsequent purification which is achieved by differential centrifugation. Morphologically recognizable cell surface structures are preserved in isolated hepatocytes for some time after preparation (at least 2 h at 4°C) as shown by electron microscopy examinations (134).

Protein phosphorylation on the outer surface of rat hepatocytes. (II).

Protein kinase activity has been shown to be present on the outer surface in a number of normal, cultured and transformed cell types (135-143). The ability of intact cells to transfer the terminal phosphate of ATP to exogenously added substrate protein (usually histones) implicates the existence of such a protein kinase activity. Cyclic AMP stimulates the surface protein kinase in some cell types, e.g. adipocytes (135), spermatozoa (137) and glial cells (141). Cyclic AMP-dependent and - independent phosphorylation of proteins was for the first time shown to occur on the outer surface of isolated rat hepatocytes in III.

Isolated hepatocytes were shown to possess protein kinase, protein phosphatase and endogenous substrates for these enzymes on the external surface. The exposed protein kinase activity was stimulated by cyclic AMP. The cyclic AMP-dependent protein phosphorylation could, at least in part, be inhibited by a protein kinase inhibitor isolated from rabbit skeletal muscle.

Control experiments were performed in order to verify that intracellular enzymes released from broken or leaking cells did not associate with the plasma membrane, thereby being able to catalyze the phosphorylation. Hydroxylapatite-agarose gel was added to the cell suspension immediately after the collagenase treatment, and was then present during the entire handling of the cells prior to phosphorylation. This treatment adsorbed approximately 80% of the protein kinase activity and cyclic AMP-binding proteins released into the medium during the preparation of hepatocytes. This did not decrease the protein kinase activity on the hepatocyte surface, which would have been expected if released enzymes became associated with the surface during the preparation.

In another control experiment we examined the incorporation of [³²P]-phosphate into soluble and particulate fractions of hepatocytes. If radioactively labelled ATP leaked into the cells, incorporation of phosphate into

soluble material would occur. The radioactivity was however recovered almost exclusively in the particulate fraction, indicating that the incorporation of phosphate into intact hepatocytes was localized to the cell surface. Kübler et al. (144) showed that histones cause HeLa cells to release cytosolic material, and they argued that histones cannot be used to detect surface-located protein kinase in any kind of cell. However, we did not observe any leakage of the cytosolic enzyme lactate dehydrogenase into the extracellular medium when isolated hepatocytes were treated with ATP, cyclic AMP and histones for short periods of time. It is therefore unlikely that the phosphorylation was due to leakage of intracellular material to the extracellular medium under our experimental conditions.

Analysis of the hepatocyte phosphoprotein pattern by SDS-polyacrylamide gel electrophoresis revealed that only a few proteins were phosphorylated by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The phosphorylation of five proteins was enhanced by cyclic AMP. A quite distinct phosphoprotein pattern with numerous phosphorylated proteins was seen when cells were homogenized or treated with Triton X-100 prior to incubation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. When EDTA was present in the incubation medium only one component became phosphorylated, namely the major phosphorylated band (M_r 50 000) obtained in the absence of cyclic AMP. The identity of the phosphorylated proteins remains to be established.

The functional importance of cell surface protein kinases and their substrates is not known. However, the addition of ATP to intact cells alters several surface dependent properties, e.g. prevents insulin-stimulated glucose transport in adipocytes (114), affects the ionic fluxes in Ehrlich ascites cells (145), and increases the permeability of transformed (but not of normal) 3T3 cells (146). There is evidence that ATP may be released from the cell in response to physiological stimuli, but the molecular mechanism for such a release is not known (147,148). It is possible that the relative concentrations of external to internal ATP may be altered by physiological stimuli. This ratio may be a factor of importance in the regulation of membrane permeability (148). Thus recent evidence indicates that a 44 000 d plasma membrane protein in fibroblasts is phosphorylated by external ATP, and that this protein is directly involved in the control of membrane permeability (149). It is therefore reason to believe that some of the diverse effects of external ATP on cells are brought about by phosphorylation-dephosphorylation of surface proteins.

Other studies suggest that there is a connection between plasma membrane-associated cyclic AMP-dependent protein kinase activity and DNA synthesis. Thus, DNA synthesis could be stimulated in T51B rat liver cells by adding either the type II holoenzyme or the catalytic subunit, but not the type I holoenzyme, to cell cultures (150). This stimulation occurred with Ca^{2+} -deprived cells, indicating a connection between cyclic AMP- and Ca^{2+} -dependent regulatory pathways. It is not clear whether the mechanism of action of the added protein kinase included an uptake of the enzyme into the cell. A cellular uptake followed by nuclear translocation has been reported for cyclic AMP-dependent protein kinases in other types of cells (151).

The cellular level of cyclic AMP has been shown to determine the degree of dissociation into subunits of cyclic AMP-dependent protein kinases. Several research groups have reported that elevation of the cyclic AMP level may result in translocation of the cyclic AMP-dependent protein kinase from the soluble fraction of the cell to nuclei or microsomes (8). In the regenerating liver the cyclic AMP level and the dissociation of the cytosolic cyclic AMP-dependent protein kinases are increased maximally at 12-14 h after partial hepatectomy. This is followed by a marked increase in nuclear protein kinase activity and a concomitant decrease in the cytosolic activity 16 h after surgery. Partial hepatectomy leads to nuclear translocation of both the type I and type II enzymes, and increased phosphorylation of histone H1 subspecies (152). Glucagon treatment of rat liver results in a translocation of the catalytic and both regulatory subunits into the nucleus (153,154). The molecular mechanism of this translocation is not known but microtubular proteins have been suggested to play a vital role (152). These studies imply that the regulation of cell proliferation and some hormonal actions may involve translocation of protein kinase subunits between intracellular compartments.

Ca²⁺- AND PHOSPHOLIPID-DEPENDENT PROTEIN PHOSPHORYLATION IN RAT LIVER SUBFRACTIONS

The Ca²⁺- and phospholipid-dependent protein kinase, called C-kinase or CaPK, has been the object of numerous investigations since its discovery in 1979 by Takai et al. (27). The C-kinase occurs ubiquitously in various tissues and phyla of the animal kingdom (28). In many tissues the activity of C-kinase far exceeds that of the cyclic AMP-dependent protein kinase (using histone H1 as phosphate acceptor protein), e.g. in rat brain, spleen and vas deferens, and in human platelets and lymphocytes (28,155). We have shown earlier (I) that a major cyclic AMP-independent protein kinase is present in rat liver cytosol and microsomes. In IV and VI we have shown that this enzyme is the newly discovered C-kinase. The cytosolic C-kinase has been partially purified and characterized (IV). Since the C-kinase was able to bind to membranes (I), we have examined some factors involved in this binding (VI). The need for a simple and sensitive procedure for analysis of membrane-bound C-kinase activity necessitated the investigation presented in V.

Cytosolic C-kinase activity (IV)

The cyclic AMP-independent enzyme described in I (fraction C I Ib) was partially purified from rat liver cytosol by DEAE-Sephacel chromatography and identified as the C-kinase by its dependency on both Ca²⁺ and phosphatidylserine for maximum enzyme activity when histone H1 was used as substrate. The C-kinase was further characterized by investigating the effects of Ca²⁺, phospholipids, histone H1, protamine, ATP and pH on the enzyme activity. The phosphorylation of protamine was five times faster than the Ca²⁺- and phosphatidylserine-dependent histone H1 phosphorylation. However, no stimulation of protamine phosphorylation was seen in the presence of Ca²⁺ and phosphatidylserine; these two substances together caused a 45% inhibition of the enzyme activity, with Ca²⁺ as the most potent inhibitor. The effect of different phospholipids on the C-kinase activity was also studied. At 0.5 mM CaCl₂ the enzyme was most active with phosphatidylserine, although other phospholipids also activated the enzyme. At low concentrations of Ca²⁺ only phosphatidylserine was stimulatory, suggesting that this phospholipid activates the enzyme in vivo. Diacylglycerol (a suggested second messenger for C-kinase (155)) was reported to be necessary together with phosphatidylserine to activate the enzyme at physiological concentrations

of Ca^{2+} . Although diacylglycerol (e.g. 1,2-diolein) did not affect the maximum activity in the presence of phosphatidylserine at 0.5 mM CaCl_2 , a stimulatory effect was seen at low concentrations of Ca^{2+} (up to 10 μM), implying that diolein might be involved in the activation of the enzyme under physiological conditions.

During the course of this work extensive purification of the soluble C-kinase from bovine heart (58) and rat brain (60), followed by a characterization of the heart enzyme (59) has been reported. The rather laborious purification procedure for the bovine heart enzyme involved ammonium sulphate fractionation, DEAE-cellulose chromatography, controlled pore glass adsorption, Sephacryl S-200 and phosphatidylserine-Affi-Gel 102 chromatographies. It resulted in 15 000 times purification of an enzyme with M_r 83 500. The enzyme was 80-95% pure. The major purification step was the affinity chromatography. The recovery was only 1%, however. The rat brain enzyme was purified 800-fold by DEAE-cellulose and Sephadex G-150 chromatographies, electrofocusing, chromatography on blue-Sepharose and phenyl-Sepharose. The recovery was 5%. The enzyme we obtain by DEAE-cellulose chromatography is purified 30-40 times over the crude cytosol compared to approximately 10 times as reported for the bovine heart and rat brain enzymes (58,60). Further purification by hydroxylapatite chromatography resulted in a 300-fold purification. Thus, by using only two steps we have obtained an enzyme purified to the same degree as the heart enzyme prior to the affinity chromatography step (58). However, our enzyme preparations have been extremely unstable and were not stabilized by addition of sucrose or glycerol (reported to stabilize the heart or brain enzymes). Therefore, the enzyme from the DEAE-Sephacel chromatography step was used for characterization. No cyclic AMP- or Ca^{2+} -calmodulin-dependent protein kinase activity could be detected in this fraction, suggesting that it was essentially free from contamination by other protein kinases. Hydroxylapatite chromatography of the C-kinase peak eluted from DEAE-Sephacel confirmed this suggestion, since virtually all protamine kinase activity overlapped with C-kinase activity. Our enzyme characteristics agreed well with those obtained with the bovine heart enzyme, implying that C-kinases obtained from different sources exhibit similar physical, kinetic and catalytic properties.

Determination of membrane-bound C-kinase activity (V).

When membrane-bound C-kinase activity is to be measured, detergent must be added to detect the enzyme activity. The detergent Triton X-100 has been used to solubilize membranes prior to measuring activity (28). However, Triton X-100 inhibits the C-kinase at concentrations above 0.0075% (156). Since this is far below what is required to solubilize membrane-bound enzymes, the extract has to be diluted extensively before the C-kinase activity can be measured. To circumvent this problem we have developed a rapid and sensitive procedure for analysis of membrane-bound C-kinase after addition of octylglycoside (n-octyl- β -D-glucopyranosid), a detergent that is tolerated well by the enzyme.

Prior to analysis membranes were solubilized in 1.75% octylglycoside. More than 95% of the microsomal C-kinase was solubilized by this treatment. The solubilized membranes were then diluted 10-fold in the C-kinase reaction mixture. No time-consuming centrifugation step to remove particulate material after solubilization prior to analysis was necessary. The C-kinase activity was obtained as the difference between the total incorporation of phosphate into histone H1 with Ca^{2+} and phosphatidylserine present and that obtained without these additions but with EGTA. The enzyme activity obtained in the presence of histone H1 and EGTA most likely represented that of the catalytic subunit of the cyclic AMP-dependent enzyme also present in membranes. Endogenous phosphorylation was not influenced by addition of Ca^{2+} and phospholipid, and was always lower than the activity obtained with histone H1 and EGTA. It was not necessary, therefore to measure endogenous phosphorylation. The conditions for analysis were optimized, i.e. the concentrations of Ca^{2+} , histone H1, Mg^{2+} , phosphatidylserine, ATP, and octylglycoside required for maximum enzyme activity were determined. Addition of phosphatidylserine was not necessary when membrane-bound C-kinase activity was measured, implying that there was a sufficiently high endogenous concentration of phospholipid in the membrane extract. The kinase activity measured, at least in microsomal extracts, represented C-kinase as shown by the quantitative recovery of a Ca^{2+} - and phosphatidylserine-dependent enzyme by DEAE-Sephacel chromatography (VI). No other Ca^{2+} -dependent protein kinase could be detected.

The highest specific activity of C-kinase was found in the N_2 plasma membrane fraction followed by the less pure P_2 fraction. Microsomes had a lower specific activity, but it was 10 times that of the cytosol. Approximately 70% of the total C-kinase activity resided in microsomes, a few per cent in plasma membranes, and the remaining portion in the cytosol. Recently, Kikkawa et al. (60) reported that only 20% of the C-kinase activity was associated with particulate material in rat liver. The discrepancy between their results and ours is probably due to their use of improper analytical conditions. They used too low amounts of detergent to solubilize the membrane properly (0.1% Triton X-100) and used 5 μ M ATP in the analysis, which is far from a saturating concentration, particularly when membrane extracts are to be analyzed.

Membrane-bound C-kinase activity (VI)

The endoplasmic reticulum is the major source of C-kinase in rat liver cells. DEAE-Sephacel chromatography of solubilized smooth microsomes revealed three protamine kinase peaks (I,VI). The major protamine-phosphorylating peak was identified as the C-kinase, since it was dependent on both Ca^{2+} and phosphatidylserine for maximum activity when histone H1 was used as substrate. Ca^{2+} -calmodulin or cyclic AMP did not activate this enzyme. The enzyme was tightly associated with the membrane, most likely on the cytosolic side, and it exhibited characteristics similar to the corresponding soluble enzyme (I).

A recent report showed that phorbol esters induce a rapid translocation of C-kinase from the cytosol to the plasma membrane in parietal yolk sac cells (157), indicating that external factors may influence the distribution of the C-kinase between soluble and particulate fractions in the cell. We have found earlier that the cyclic AMP-independent protein kinase of the cytosol (fraction C IIb), now identified as the C-kinase (IV), could bind to smooth microsomes (I). However, at the time we did not examine further which factors were involved in this binding. Takai et al. (29) postulated that the soluble C-kinase is able to bind reversibly to membranes in the presence of Ca^{2+} . No experimental support for this suggestion was given, however. Our experiments showed that the soluble C-kinase would bind to Ca^{2+} -depleted microsomes after addition of Ca^{2+} . Maximally, about five times more C-kinase bound than the activity usually found in microsomes. Mg^{2+} could not substitute for Ca^{2+} . A rather high concentration of Ca^{2+} (100 μ M) was needed how-

ever, for binding to occur. This concentration is much higher than the concentration found in the soluble portion of the cell, but the local concentration of Ca^{2+} at membrane surfaces may be rather high. Diolein has been reported to increase the apparent affinity of the C-kinase for Ca^{2+} and phospholipid so that binding would occur at physiological concentrations of Ca^{2+} (29). Diolein is usually almost absent in membranes, but it can be produced transiently by phosphatidylinositol breakdown induced by certain physiological stimuli. Neither diolein added extrinsically nor diacylglycerol generated intrinsically in the microsomes (by a phospholipase C specific for phosphatidylinositol (158)) affected the binding of C-kinase to these membranes at various concentrations of Ca^{2+} , suggesting that diolein is not necessary for binding.

Removal of Ca^{2+} from microsomes by treatment with EGTA did not release C-kinase. Approximately 50% of the bound C-kinase in the recombination experiments resisted EGTA treatment resulting in a 2-fold excess of C-kinase irreversibly bound. This suggests that the binding is Ca^{2+} -dependent, but that the release of C-kinase from membranes is determined by other factors than Ca^{2+} . However, more work is required to elucidate the molecular basis for interactions between C-kinase and membranes.

The physiological role of soluble and membrane-bound C-kinase in liver cells is not known, since no physiological substrates have been identified. Human fibrinogen has been shown to be phosphorylated by C-kinase in vitro (159). In platelets, a 40 000 d protein becomes phosphorylated upon stimulation with thrombin, and this phosphorylation is accompanied by a release of dense body constituents, such as serotonin (30). The isolated 40 000 d protein can serve as substrate for C-kinase which phosphorylates the same site as is phosphorylated in vivo (30). This suggests that C-kinase is involved in platelet activation. Furthermore, recent experiments have shown that C-kinase copurifies with the phorbol diester receptor (160). If the receptor is identical to C-kinase, an important physiological role for membrane-bound C-kinase in the control of cellular activities is at hand.

As mentioned earlier C-kinase became translocated from the cytosol to plasma membranes by treatment of parietal yolk sac cells with tumor-promoting phorbol esters (157). The enzyme could not be extracted by salt or EDTA/EGTA treatment, suggesting that it had become tightly associated with the plasma membrane. Phorbol esters can activate C-kinase in the

presence of phospholipid and low concentrations of Ca^{2+} , thus substituting for diacylglycerol. While the life-time of diacylglycerol in the membrane is very short, phorbol esters intercalate with the phospholipids and are metabolized slowly. Thus, they can keep the C-kinase active for a prolonged period of time. Whether this has any significance for their tumor-promoting properties remains to be elucidated.

SUMMARY

The cyclic AMP-dependent type I protein kinase was found in the soluble portion of rat liver, while the type II enzyme was distributed between cytosol and membranes. Since these protein kinases differ in their regulatory subunits it is likely that the regulatory subunit determines whether the enzyme will interact with membranes or not. The free catalytic subunit of these kinases was present in all three subfractions examined.

The cyclic AMP-independent protein kinase present in cytosol (fraction C IIb) and microsomes (fraction M II) was identified as the newly discovered Ca^{2+} - and phospholipid-dependent protein kinase, termed C-kinase. This enzyme was also present in plasma membranes. Partially purified C-kinase bound tightly to microsomes in the presence of Ca^{2+} . The bound enzyme could not be released from the membranes by treatment with salt or EGTA. This suggests that Ca^{2+} is necessary for binding to occur, but not for the retention of the enzyme on the membrane.

The transverse orientation of membrane-bound protein kinases was investigated. The C-kinase is probably located at the cytoplasmic side of microsomal membranes since it is susceptible to protease attack, whereas the type II cyclic AMP-dependent protein kinase is present on the luminal side. In plasma membranes the type II enzyme presumably is located on the external surface. The physiological significance of the orientation of these enzymes is still a matter for speculation.

The membrane-bound protein kinases had similar catalytic, physical and kinetic properties as the corresponding soluble ones, suggesting that the enzymes do not undergo substantial changes upon binding to membranes. The membrane-bound protein kinase activity measured with protamine as substrate comprises approximately 30% of the total activity in rat liver. 70% of the total C-kinase activity is associated with membranes, and most of this with microsomes.

These results provide a basis for future studies of membrane-bound protein kinases and their function within the cell. It will be of interest to investigate which factors influence the distribution of protein kina-

ses between different cellular compartments and the physiological significance of enzyme translocation. The identification of membrane target proteins for the protein kinases is necessary for an understanding of the physiological function of these enzymes in different cellular compartments. Today, only few substrates for the cyclic AMP-dependent protein kinases are known, whereas no physiological substrate has been identified for the C-kinase in liver.

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Protein Kinases of Rat Liver Endoplasmic Reticulum

Solubilisation, Partial Characterisation and Comparison with Protein Kinases of Rat Liver Cytosol

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Protein kinase associated with rat liver microsomes was only partly extracted by treatment with 1.5 M KCl. The enzyme was solubilised by Triton X-100 or sodium deoxycholate at the same or slightly higher detergent concentrations than microsomal marker components. The enzyme activity increased 2–3-fold upon solubilisation. Three peaks with protein kinase activity (fractions MI, MII and MIII) were resolved on DEAE-cellulose chromatography. Fraction MIII but not fractions MI or MII was activated by adenosine 3':5'-monophosphate (cyclic AMP). All fractions catalysed the phosphorylation of protamine and histones but not that of casein or phosphovitin. Fractions MI and MIII had a similar substrate specificity and phosphorylated histones at a relatively much higher rate than did fraction MII. The isoelectric points were 8.1 for fraction MI, 5.5 for fraction MII and 4.9 for fraction MIII. On incubation of fraction MIII with cyclic AMP it was split into two catalytically active components with pI 8.1 and 7.35. The component with pI 8.1 was predominant and corresponded to fraction MI. Five protein kinase peaks were resolved from rat liver cytosol by DEAE-cellulose chromatography. Three of them (fractions CIa, CIb and CIII) had the same properties as each of the microsomal kinase fractions. A fourth fraction (CIIa) was cyclic-AMP-dependent and had the same substrate specificity as fractions MI and MIII. Its pI was 5.1, and it was split into two components by cyclic AMP (pI 8.1 and 7.35). In binding studies fraction CIb bound more efficiently to microsomes than fraction CIII, while fractions CIa, CIIa and the microsomal protein kinase fractions did not bind appreciably. When microsomes were treated with trypsin exposed protein kinase was inactivated and the latency of the remaining enzyme increased substantially. Most of fraction MII was inactivated by trypsin while fraction MIII was resistant. The possible orientation of protein kinase fractions MII and MIII in the microsomal membrane is discussed.

Protein kinases are present in the cytosol as well as in various particulate fractions of several tissues (for a review, see [1]). In rat liver cytosol there are both cyclic-AMP-dependent and independent forms of the enzyme [2,3] which presumably participate in the regulation of various cellular activities. A major function of cyclic-AMP-dependent protein kinases, for instance, appears to be as an intracellular link in hormone action [4]. Several protein substrates for protein kinases are present in rat liver cytosol [5], as well as in other subcellular fractions [6]. Specifically enzymes involved in glycogen metabolism (for a review, see [7]), pyruvate kinase [8], histones [9] and acidic nuclear proteins [10] have been shown to

undergo phosphorylation reactions which in some cases are enhanced by cyclic AMP.

We have previously found [6] that rat liver endoplasmic reticulum contains protein kinase activity and that microsomal proteins can be phosphorylated endogenously by this enzyme. A part of the protein kinase was found to be loosely associated with the membrane and could be removed by salt treatment, while the remaining enzyme was more tightly bound and was solubilised by detergent treatment. The tightly bound enzyme appeared to be partly hidden in the membrane since its activity increased 2–3 times upon solubilisation.

This investigation is focused on the binding of protein kinase to rat liver microsomes and its position in the membrane. The microsomal enzyme has been resolved into three fractions which are partly characterised and their properties are compared to those of protein kinases present in rat liver cytosol.

Abbreviation. Cyclic AMP, adenosine 3':5'-monophosphate.

Enzymes. Cytochrome b_5 reductase or NADH: cytochrome b_5 (ferricyanide) oxidoreductase (EC 1.6.2.2); protein kinase or ATP: protein phosphotransferase (EC 2.7.1.37).

MATERIALS AND METHODS

Materials

[γ - 32 P]ATP and cyclic [8- 3 H]AMP were obtained from The Radiochemical Centre (Amersham) and cyclic AMP, ATP, protamine, α -casein, phosvitin and trypsin (bovine pancreas, type III) from Sigma. Ampholine was purchased from Pharmacia. Calf thymus histone H1 and trout testis histones H2b, H3 and H4 were prepared as described earlier [11].

Methods

All operations were performed at 0–4 °C unless otherwise is indicated. Livers were obtained from male Sprague-Dawley rats (200–300 g) fed *ad libitum* and microsomes were prepared as described earlier [12]. Usually, the microsomes were fractionated by sucrose gradient centrifugation to yield smooth microsomes at the 0.9–1.3 M sucrose interface. Before use both microsomes and the smooth fraction were washed by suspension in standard buffer (50 mM Tris-HCl, pH 7.4; 5 mM MgCl₂; 1 mM dithioerythritol) also containing 0.3 M KCl and resedimentation at 110 000 $\times$ g for 90 min. The pellets obtained were suspended in buffer with the aid of an all-glass Potter-Elvehjem homogeniser.

Subcellular Fractionation

To examine the subcellular distribution of protein kinase activity rat livers weighing 12–13 g were homogenised in 30 ml of buffer also containing 0.35 M sucrose in a homogeniser fitted with a teflon pestle. After passing through four layers of gauze the homogenate was fractionated according to the scheme presented in Fig. 1.

Extraction of Protein Kinase from Microsomes

Smooth microsomes (or in some cases unfractionated microsomes) suspended in buffer were treated with 2% (w/v) Triton X-100. After 30 min incubation at 0 °C the extracts were centrifuged at 110 000 $\times$ g for 90 min and any pellet was discarded.

In an extraction experiment with deoxycholate and Triton X-100 the suspension of smooth microsomes was diluted with buffer to a membrane protein concentration of 5.9 mg/ml. 250 μ l of the suspension was mixed with 250 μ l of sodium deoxycholate or Triton X-100 of various concentrations. The Triton solutions also contained 0.6 M KCl. After incubation for 30 min at 0 °C the samples were centrifuged at 105 000 $\times$ g for 90 min and the pellets obtained were suspended in standard buffer.

In another extraction experiment with KCl and deoxycholate 500 μ l of a suspension of smooth

microsomes (protein concentration 6.9 mg/ml) was treated with KCl of various concentrations with or without deoxycholate in a final volume of 4 ml. The samples were sonicated for 6 $\times$ 10 s at 0 °C using a Branson sonifier fitted with a microtip at 45 W. After centrifugation at 105 000 $\times$ g for 90 min pellets were suspended in standard buffer.

DEAE-Cellulose Chromatography

A 1.4 $\times$ 25-cm column was packed with DE-52 (Whatman) and equilibrated with 10 mM Tris-HCl, pH 7.4, 1 mM MgCl₂ and 0.1 mM dithioerythritol. Samples were dialysed against the same buffer prior to chromatography. After application of a sample the column was washed with buffer and then developed with a linear salt gradient running from 0 to 0.4 M KCl in a total volume of 500 ml buffer. Flow rate was 25 ml/h and fractions of 5 ml were collected. Protein kinase peak fractions were combined as indicated in figures and dialysed against the chromatography buffer and concentrated in Minicon concentrators (Amicon).

Isoelectric Focusing

Isoelectric focusing was performed according to the principle described by Vesterberg and Svensson [13]. Two glass tubes (their size was 1.4 $\times$ 30 cm) mounted vertically were used as separation chamber and electrode vessel, respectively. They were connected at the bottom by a V-shaped glass tube fitted with a three-way valve for filling and emptying of the apparatus. The separation tube had a cooling jacket through which water of 0 °C was pumped. The sample (0.5–1 ml) was mixed with 10% sucrose and 50 μ l 40% Ampholine (usually pH range 3–10). This mixture together with 40% sucrose supplied with 250 μ l Ampholine was used to form a linear 10–40% sucrose gradient in the separation tube. The total volume of the gradient was 22 ml and it also contained 0.1 mM dithioerythritol. The electrode tube and the bottom of the separation tube were filled with 1% sulphuric acid in 50% sucrose as anode solution and 2% ethanolamine was layered on top of the gradient as cathode solution. The focusing time was 36–40 h, starting with a voltage of 600 V (maximum current 3 mA) which was increased to 1200 V after 1–2 h. After the end of a run the sucrose gradient was collected into fractions of 300–400 μ l. The pH of fractions was measured at 4 °C in a Radiometer BMS 3 micro analyser. In some instances, samples were incubated with 0.1 mM cyclic AMP and 10 mM theophylline for 15 min at 20 °C prior to the focusing to dissociate cyclic-AMP-dependent protein kinase. 0.1 mM cyclic AMP was then included in the sucrose gradient. The yield of protein kinase activity after isoelectric focusing was usually 30–40%.

Trypsin Treatment

Smooth microsomes (protein concentration 4.2 mg/ml) were incubated with trypsin (the protease: membrane protein ratio was 1:50) for 12 h at 0 °C. The reaction was stopped by the addition of soybean trypsin inhibitor (3 times the amount of trypsin). In control incubations the inhibitor was added before trypsin to the sample or trypsin treatment was performed in the presence of 2% Triton X-100. To examine whether active protein kinase was removed from the membrane by trypsin an aliquot of each reaction mixture was centrifuged at $105\,000 \times g$ for 90 min and the resulting pellet was suspended in standard buffer before analysis of supernatant and suspension.

Binding Studies between Protein Kinases and Smooth Microsomes

Protein kinases from microsomes and the cytosol were prepared by DEAE-cellulose chromatography and concentrated as described above. The cytosolic protein kinase fraction CIIb was rechromatographed under the same conditions before concentration to remove contaminating fraction CIIa and cyclic-AMP-binding proteins. Various amounts of each protein kinase fraction were incubated with smooth endoplasmic reticulum (0.59 mg membrane protein) at 0 °C in 1 ml of 10 mM Tris-HCl, pH 7.4, 1 mM MgCl₂ and 0.1 mM dithioerythritol. After 15 h the samples were diluted to 6 ml with the buffer and KCl was added to a final concentration of 0.3 M. After incubation for 1–2 h at 0 °C the samples were centrifuged for 90 min at $105\,000 \times g$. The supernatants were removed and the surface of the pellets was washed with buffer before suspension in 400 µl of the above buffer with a glass-teflon homogeniser. Protein kinase activity of the suspensions was measured in the absence or presence of 5 µM cyclic AMP and 2% Triton X-100 with protamine as substrate [11].

Analyses

Protein kinase activity was measured as described earlier [11] with protamine as substrate. When present, cyclic AMP was added to a final concentration of 5 µM. In some cases samples were treated with 2% (w/v) Triton X-100 before addition of the reaction mixture.

Cyclic-AMP-binding analyses were performed using a slight modification [6] of the procedure described by Gilman [14]. Cytochrome *b₅* and cytochrome *P*-450 were determined according to Omura and Sato [15,16], and NADH-ferricyanide reductase according to Hrycay and O'Brien [17]. Protein was determined by the method of Lowry et al. [18] after

precipitation in 10% trichloroacetic acid at 0 °C with bovine serum albumin as standard.

RESULTS

Subcellular Distribution of Rat Liver Protein Kinase

To get an estimate of the subcellular distribution of protein kinase activity rat livers were fractionated according to the scheme in Fig. 1. Protein kinase was measured in the absence and presence of cyclic AMP and Triton X-100 with protamine as substrate (Fig. 2). The total soluble kinase activity obtained by the addition of the enzyme activity of the cytosol and that of the washes of the particulate fractions (which appear to contain enzyme that is loosely bound to cell particles) comprised approximately 2/3 of the total activity in liver. The remaining enzyme was distributed between nuclei, mitochondria, rapidly sedimenting endoplasmic reticulum [19] and microsomes. The protein kinase activity of microsomes was around 10% of the total enzyme varying slightly depending on analysis conditions. The yield of enzyme in the subcellular fractions was close to 200% when compared to the activity of the homogenate, while the yield of protein was 100%. The cytosolic and nuclear protein kinase was particularly stimulated by cyclic AMP, while a slight stimulation was seen in the other fractions. An enhancement in enzymic activity was also obtained in the presence of Triton X-100. The enhancement was most prominent in microsomes and rapidly sedimenting endoplasmic reticulum where it was approximately 2-fold in line with our previous finding that microsomal protein kinase activity is partly hidden in the membrane and can be released upon solubilisation of the membrane [6]. The enzyme still bound to mitochondria, rapidly sedimenting endoplasmic reticulum and microsomes after washing in buffer containing 0.3 M KCl appeared to be rather firmly attached since a second wash in the same medium removed very little enzyme. However, the treatment tended to diminish the stimulatory effect of cyclic AMP (cf. [6]).

The subcellular distribution of protein kinase obtained by us is in general agreement with that of earlier reports [3,20]. We found, however, a much smaller proportion of the total kinase in the soluble fraction than Chen and Walsh [3], but a larger proportion in this fraction than Rousseau et al. [20]. This might in part be due to our use of protamine rather than various histones as a substrate. Protamine appears to be a more general substrate for protein kinase than histones (cf. below) probably due to the presence of different amino acid sequences around phosphorylatable seryl groups [21,22] which ap-

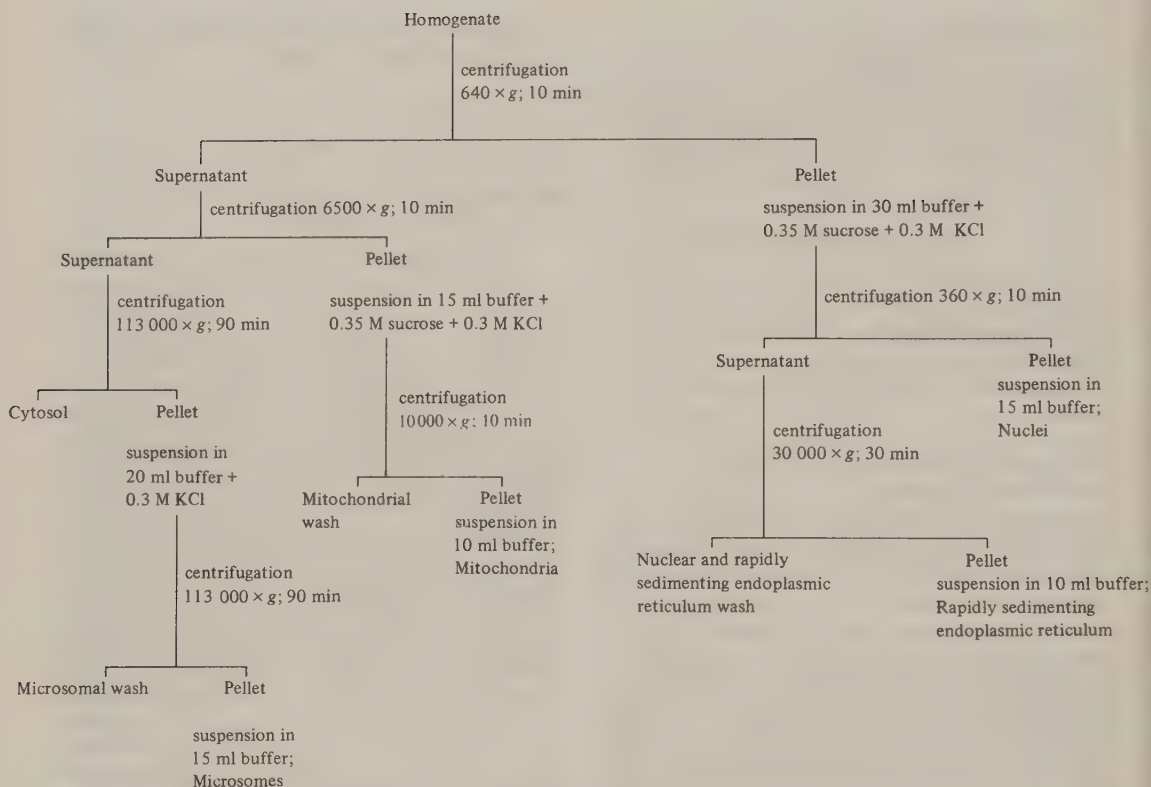


Fig. 1. Scheme for the fractionation of rat liver. Subcellular fractions which were analysed further are underlined. The buffer used was the standard buffer described in the Methods section

parently closely resembles the optimal sequence for phosphorylation recognised by various protein kinases [23].

Extraction of Protein Kinase from Microsomes

To examine how tightly the microsomal protein kinase was bound to the membrane extraction experiments were performed using smooth microsomes as the source of enzyme. In a first set of experiments smooth microsomes were sonicated under various salt and detergent conditions and the distribution of protein kinase and microsomal marker components between solubilised and membrane material was measured (Table 1). Only little protein kinase or microsomal markers were solubilised by sonication in the presence of 0.3 M KCl. At 1.5 M KCl more protein kinase than markers was released, but still 65% of the enzyme remained attached to the particulate material. A second treatment with 1.5 M KCl released the same proportion of protein kinase and markers as the first treatment. Sonication of microsomes in the presence

of 0.1% deoxycholate and 1.5 M KCl has been reported to extract all proteins except integral ones [24]. This treatment released practically all protein kinase as well as integral marker components, while around 20% of the protein was left in the particulate material. A less drastic treatment with 0.1% deoxycholate and 0.3 M KCl solubilised two thirds of both protein kinase and protein from the membrane together with half of the cytochrome b_5 and most the NADH reductase and cytochrome $P-450$. Extraction with deoxycholate alone (0.05% was used in this experiment, but the extraction is not changed appreciably if the concentration is increased to 0.1%, see Fig. 3) left most of the protein, protein kinase and markers in the particulate fraction. Thus, protein kinase appears to be less resistant to salt extraction than microsomal marker components. On the other hand, some of the markers are more easily extracted than protein kinase in the presence of both KCl and the ionic detergent deoxycholate. The cytosolic marker enzyme lactate dehydrogenase was present in insignificant amounts on the microsomes used for the extraction experiments.

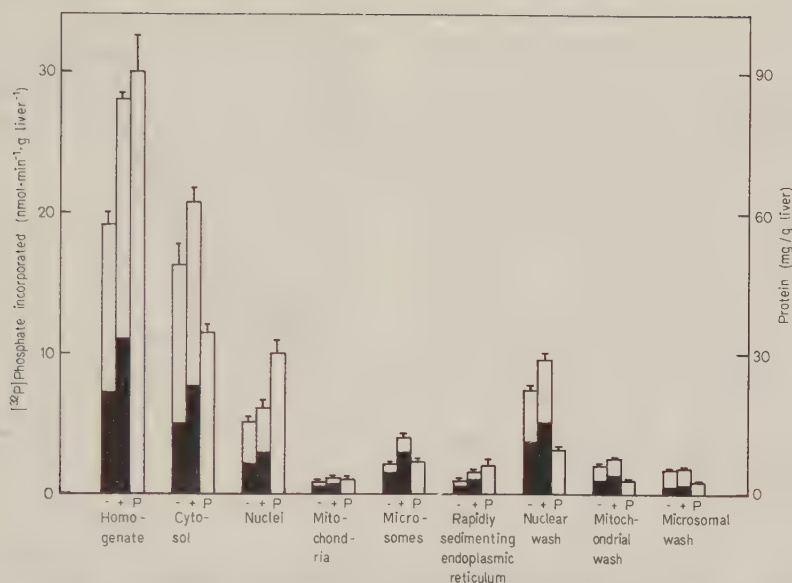


Fig. 2. Subcellular distribution of protein kinase in rat liver. Rat livers were fractionated according to Fig. 1 and the fractions obtained were analysed for protein kinase activity using protamine as substrate and for protein content. The first bar (—) represents the activity in the absence of detergent, and the second bar (+) the activity obtained in the presence of 2% Triton X-100. The dark area is the activity without cyclic AMP, while the whole bar represents the activity in the presence of 5 μ M cyclic AMP. S.E.M. values obtained from three different liver preparations are given. Activities are expressed as [32 P]phosphate incorporated at 20 $^{\circ}$ C in $\text{nmol} \times \text{min}^{-1} \times \text{g liver}^{-1}$. The third bar (P) represents protein content (mg/g liver) with S.E.M. values

Its specific activity was around 1% and its total activity was less than 0.1% of that found in the cytosolic fraction.

The solubilisation of protein kinase compared to microsomal marker components was also examined using various concentrations of the ionic detergent deoxycholate or the nonionic detergent Triton X-100 (Fig. 3). Protein kinase tended to solubilise at a higher detergent concentration than either of the cytochromes b_5 and P -450 or NADH reductase. This tendency was particularly evident with Triton X-100. At 0.5% Triton X-100, for instance, half of the protein kinase activity still remained bound to particulate material, while 75% or more of the three microsomal marker components had been released. The markers and protein kinase were more resistant to extraction than the bulk of proteins originally associated with the membrane.

There was a 2-fold increase in protein kinase activity upon addition of detergent to microsomes. This increase appeared at 0.1% deoxycholate (cf. Table 1) and between 0.08% and 0.15% Triton X-100, detergent concentrations which were not sufficient to solubilise the enzyme. Since we have found that purified membrane protein kinases are not stimulated significantly by Triton X-100, we conclude that the increase in activity was due to the fact that the mem-

brane-bound enzyme was partly inaccessible to the protein substrate unless the structure of the membrane had been disturbed by the addition of detergent.

DEAE-Cellulose Chromatography

Protein kinase bound to smooth microsomes was solubilised by Triton X-100 and subjected to DEAE-cellulose chromatography. Three fractions with kinase activity (labelled MI, MII and MIII) were resolved (Fig. 4A). An extract of rough microsomes yielded a similar protein kinase pattern as smooth microsomes. In some instances when a small amount of material was chromatographed, fraction MIII resolved into two peaks. None of the protein kinase fractions was activated by cyclic AMP when eluted from the column, but after concentration fraction MIII became partly dependent on the cyclic nucleotide for maximal activity (see Table 3). It is likely that the comparatively high salt concentration in combination with the rather low enzyme concentration in the column eluate keeps the enzyme in a dissociated and thereby fully activated state similarly to what has been found for the type II protein kinase of mouse liver cytosol [25].

When rat liver cytosol was chromatographed under the same conditions as the microsomal extract five protein kinase fractions were resolved (Fig. 4B). Two

Table 1. Extraction of protein kinase and microsomal markers from smooth microsomes with salt. Smooth microsomes were treated with KCl and sodium deoxycholate as described in the text. Analyses were performed on extracts and unextracted material (pellets) and the distribution of the various activities between these fractions was determined. Yields are expressed relative to untreated microsomes. Protein kinase activity is expressed as ^{32}P incorporation.

Extraction condition	Protein kinase			Protein			Cytochrome b_5			Cytochrome P-450			NADH-ferricyanide reductase		
	nmol ^{32}P incorporated per min	%	mg	extract pellet	distri- bution, extract- pellet	yield	extract pellet	nmol heme	%	extract pellet	distri- bution, extract- pellet	yield	extract pellet	distri- bution, extract- pellet	yield
Untreated	1.26		100	3.60	17-83	100	1.94			2.53		100	18.6		100
0.3 M KCl	0.22	16-84	106	0.59	2.92	98	0.36	1.62	18-82	0	2.74	102	1.8	9-91	104
1.5 M KCl	0.58	35-65	131	0.63	2.92	99	0.26	1.72	13-87	0	3.50	102	1.6	10-90	90
0.3 M KCl + 0.1% deoxycholate	1.59	66-34	190	2.18	1.06	90	0.64	0.78	45-55	73	5.56	0	18.0	87-13	112
1.5 M KCl + 0.1% deoxycholate	2.61	89-11	233	2.66	0.66	92	1.56	0.22	88-12	92	4.52	0.18	16.5	95-5	94
0.05% deoxycholate	0.49	31-69	126	1.00	2.48	97	0.26	1.30	17-83	80	0.91	3.22	3.7	15.5	103

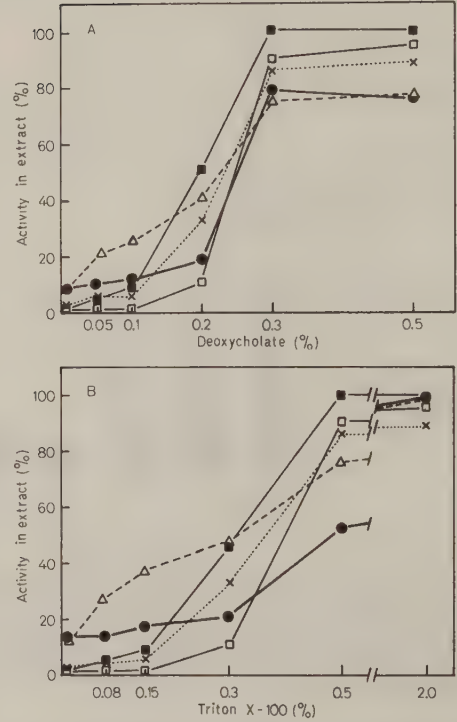


Fig. 3. Extraction of protein kinase and microsomal markers from smooth microsomes with detergents. Smooth microsomes were treated with various concentrations of sodium deoxycholate or Triton X-100 + 0.3 M KCl as described in the text. Analyses were performed on extracts and unextracted material and the distribution of the various activities between these fractions was determined. The activity recovered in extracts (in % of total recovered activity) is plotted against detergent concentration. (A) Extraction with sodium deoxycholate; (B) extraction with Triton X-100 + 0.3 M KCl. (●) Protein kinase; (□) cytochrome b_5 ; (■) cytochrome P-450; (×) NADH-ferricyanide reductase; (Δ) protein

of them (fractions CIIa and CIII) were activated, although to a different degree, by cyclic AMP and appear to be the type I and type II protein kinases, respectively, as defined by Corbin et al. [26]. Fraction CIII eluted as a rather broad peak which in some instances resolved into two peaks similarly to the corresponding microsomal enzyme fraction. Fractions CIa and CIb (the latter hidden in the ascending part of fraction CIIa) and fraction CIIb were not activated by cyclic AMP. When the elution patterns of microsomes and cytosol were compared the microsomal protein kinase fractions MI, MII and MIII were found to elute at the same KCl concentrations (0.03, 0.11 and 0.20 M) as the cytosol fractions CIa, CIIb and CIII, respectively.

We also examined the chromatographic pattern of protein kinase that was released from smooth micro-

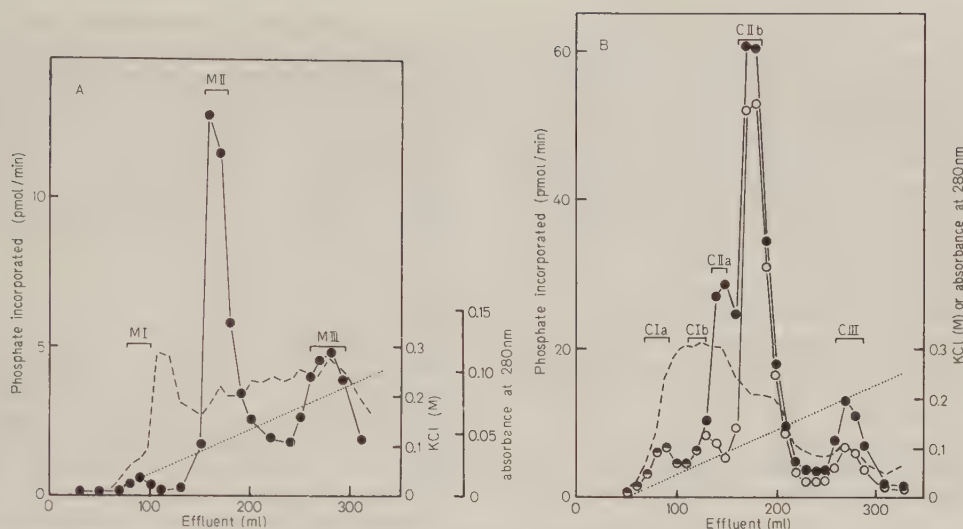


Fig. 4. Fractionation of protein kinases by DEAE-cellulose chromatography. Conditions were as described in the Methods section. Protein kinase analyses were performed in the presence (●) or absence (○) of cyclic AMP. (---) Absorbance at 280 nm; (····) KCl gradient. (A) Smooth microsomes solubilised by Triton X-100; (B) rat liver cytosol. The applied amounts of protein were 55 mg and 500 mg, respectively. The kinase activity of the eluate of smooth microsomes was independent of cyclic AMP

somes upon sonication in the presence of 1.5 M KCl. Enzyme peaks eluting in the same positions as the microsomal protein kinase fractions MI and MIII were obtained, while no enzyme eluted in the position of fraction MII. The peak in the MI position was larger than that of fraction MIII most likely due to a dissociation of fraction MIII into regulatory and catalytic subunits under the extraction conditions used. There was also more fraction MI than fraction MIII left in the membrane material after the extraction supporting the idea of a dissociation of fraction MIII. Fraction MII, on the other hand, appeared to be unaffected by the salt and remained bound to the membrane upon salt treatment.

Earlier work by others has shown that the protein kinase activity of rat liver cytosol can be resolved into several peaks on chromatography using DEAE-cellulose or DEAE-Sephadex [3,26–29]. The results differ, however, due to the different experimental conditions used, and probably particularly due to the different histone preparations used as phosphate acceptors in the protein kinase reaction. The introduction of a 'standard set' of protein or peptide substrates for protein kinases would facilitate a comparison of results from different laboratories. The elution patterns obtained by some workers [3,26,29] are, however, similar to our pattern although the major cyclic-AMP-independent protein kinase is missing in their chromatograms since histones are inefficient substrates for this enzyme fraction (see below).

Uno et al. [30] resolved protein kinase from both cytosol and the 150000 × *g* pellet of bovine liver into two cyclic-AMP-dependent enzymes by DEAE-cellulose chromatography. One enzyme from each of these subcellular fractions eluted at the same low salt concentration, while the other enzymes showed different elution properties. Since all enzyme fractions were stimulated by cyclic AMP the enzymes eluting at low salt most likely corresponded to our cytosol protein kinase CIIa (which might not have been removed completely from the membrane by their sucrose washes). The bovine enzymes eluting at higher salt concentrations should correspond to our fractions MIII and CIII, although their elution properties were not the same which was the case for the two rat liver enzymes. An enzyme corresponding to our fraction MII (or CIIb) cannot be detected in the chromatograms of Uno et al. [30] probably due to the fact that such an enzyme should be rather inactive with histones as substrate (see below).

Isoelectric Focusing

The protein kinase fractions from microsomes and cytosol separated by DEAE-cellulose chromatography were examined by isoelectric focusing in the presence or absence of cyclic AMP (Table 2). The *pI* values of the microsomal fractions MI, MII and MIII were 8.1, 5.5 and 4.9, respectively. Corresponding enzymes from the cytosol (fractions CIa, CIIb, and CIII) showed

similar isoelectric points, and each of them comigrated with its microsomal counterpart. The cytosolic enzyme CIIa had the *pI* 5.1. After treatment of each of the cyclic-AMP-dependent protein kinase fractions (MIII, CIIa and CIII) with cyclic AMP two enzymically active peaks were resolved, a more prominent peak comprising approximately 30% of the enzyme activity with *pI* 8.1 and a smaller one with *pI* 7.35. The *pI* 8.1 peak comigrated with fractions MI and CIa, and we have evidence that the *pI* 7.35 peak corresponds to fraction CIb. Fraction CIII was rather resistant to dissociation by cyclic AMP and undissociated protein kinase was seen in the separation involving this fraction. The *pI* of the cyclic-AMP-independent enzyme fractions (MI, MII, CIa and CIIb) was not affected by the nucleotide.

The appearance of two catalytically active peaks on isoelectric focusing of cyclic-AMP-dependent protein kinase after treatment with the nucleotide was

Table 2. *Isoelectric points of protein kinase fractions*

Isoelectric focusing was performed as described under Methods. Samples were run without cyclic AMP treatment or after pretreatment with 0.1 mM cyclic AMP and 10 mM theophylline for 15 min at 20 °C

Protein kinase fraction	Isoelectric point	
	no cyclic AMP	+ cyclic AMP
MI	8.05	8.05
II	5.50	5.50
MIII	4.80	8.10 7.35
CIa	8.10	8.15
CIIa	5.10	8.15 7.35
CIIb	5.45	5.45
CIII	4.90	8.15 7.35

first observed by Chen and Walsh [3]. Their results also indicate that the *pI* of the cyclic-AMP-dependent enzyme differs slightly from that of the dissociated enzyme peak having the higher *pI*. This is in contrast to our finding that the cyclic-AMP-independent protein kinase of *pI* 8.1 (fraction CIa or MI) focuses together with the major enzymically active component obtained by treatment of the cyclic-AMP-dependent kinases with the nucleotide.

Substrate Specificity

The substrate specificity of the various protein kinase fractions from microsomes and cytosol was examined (Table 3). The activities of various substrates are expressed relative to the activity of protamine analysed in the presence of 0.3 M NaCl. The enzyme fractions MI, MIII, CIa, CIIa and CIII all have a similar specificity in the presence of cyclic AMP with a comparatively high rate of phosphorylation of histones H1 and H2b. However, the degree of activation by cyclic AMP differs for the enzymes, being approximately 10-fold for fraction CIIa (with protamine as substrate) and 2-fold for fractions MIII and CIII, while the activity of fractions MI and CIa was not affected by the nucleotide. The presence of 0.3 M NaCl in the incubation medium was also necessary for full activity of these enzyme fractions when protamine was the substrate. The fractions MII and CIIb phosphorylated the various histones at a low rate, the phosphorylation of protamine was not affected by salt and they could not be activated by cyclic AMP.

These results, together with the chromatographic and isoelectric focusing evidence, indicate the presence in both microsomes and cytosol of two types of protein kinases using protamine as substrate which have different catalytic properties. One type is represented by fractions MII and CIIb which appear to be similar or

Table 3. *Substrate specificity of protein kinases*

Substrate specificities of protein kinases from smooth microsomes and cytosol purified through the DEAE-cellulose chromatography step were determined with or without 5 μ M cyclic AMP. Activities for various substrates are expressed relative to the activity towards protamine in the presence of cyclic AMP and 0.3 M NaCl. —, no cyclic AMP; +, with cyclic AMP

Substrate	Microsomal protein kinase						Cytosolic protein kinase							
	MI		MII		MIII		CIa		CIIa		CIIb		CIII	
	—	+	—	+	—	+	—	+	—	+	—	+	—	+
Histone H 1	42	34	7	12	10	39	25		21		5		25	
H 2b	81	65	2	3	12	67	58	60	6	51	17		6	68
H 3		22	1	2	1	5		9		6	3			11
H 4		10	1	0	2	1		14		3	2			4
Protamine — NaCl	47	46	100	114	44	62	45	47	6	40	122	112	42	57
Protamine + NaCl	103	100	96	100	68	100	106	100	10	100	85	100	47	100
Casein	9	9	1	0	1	1	1		0		1			2
Phosvitin	9	9	0	0	1	1	0		0		1			2

identical enzymes. They are not affected by cyclic AMP and do not combine with free regulatory subunits (unpublished results). Fractions MIII and CIII together with fraction CIIa represent a second type which is cyclic-AMP-dependent. They are composed of the same (or similar) two catalytic components, one of which most likely corresponds to fraction MI or CIIa, while fraction CIIb is the other. These components will also combine with free regulatory subunits to form cyclic-AMP-dependent enzymes. Fractions CIIa and CIII apparently differ in their regulatory part. There is evidence, for instance, that the regulatory components of type II protein kinases [26], probably corresponding to our fraction CIII, may undergo phosphorylation [31]. The difference between fractions CIIa and CIIb is not clear. Although they differ in their chromatographic and isoelectric properties they have a similar substrate specificity (unpublished results).

Effect of Trypsin Treatment on Microsomal Protein Kinase

The partial latency of microsomal protein kinase activity observed as a stimulation of the enzyme upon detergent treatment, suggests that one or more of the microsomal protein kinase fractions might be hidden in the membrane. To examine this matter further, the effect of trypsin treatment on microsomal protein kinase activity was tested. Analyses were performed in the absence of Triton X-100 to monitor kinase activity presumed to be on the membrane surface and in the presence of the detergent to monitor the total kinase activity in the membrane.

After trypsinisation only 20% remained of the protein kinase activity originally present in untreated microsomes when monitored without Triton X-100 (Table 4). In contrast, more than 50% of the activity remained if analyses were performed in the presence of the detergent. Thus, the stimulatory effect of Triton X-100 on kinase activity increased from 3 to more than 7 times due to trypsin treatment. However, the difference in activity between untreated and treated samples was only slightly higher when kinase was analysed in the presence of Triton X-100 than when analysed without detergent. These results are consonant with a selective proteolytic removal of protein kinase activity exposed on the surface of microsomes, while hidden enzyme is protected against the treatment.

It is also seen in the table that most of the protein kinase activity remained bound to the particulate fraction (pellets) and that very little activity was found in the supernatant after centrifugation of the suspensions. Thus, catalytically active protein kinase was not released from the microsomes by proteolytic treatment, as has been shown to be the case for cytochrome b_5 and other microsomal enzymes [32]. The control

Table 4. *Effect of trypsin treatment on protein kinase activity of smooth microsomes*

Smooth microsomes were prepared and treated as described under Methods. Aliquots of the treated suspensions were centrifuged to produce supernatant and pellet (which was suspended in the same amount of buffer). Protein kinase analyses were performed in the presence or absence of 2% Triton X-100 using 20 μ l of sample. Activity is expressed as 32 P incorporation

Treatment	Fraction	Protein kinase activity		
		– Triton X-100	+ Triton X-100	Ratio, with/with- out Triton X-100
pmol/min				
Untreated	suspension	23.5	65.7	2.8
	supernatant	2.2	2.7	
Trypsin	pellet	22.6	64.9	2.9
	suspension	4.8	36.7	7.6
	supernatant	1.1	1.3	
Soybean trypsin inhibitor + trypsin	pellet	5.1	35.2	6.9
	suspension	22.7	69.6	3.1
	trypsin + 2% triton X-100		2.6	

experiment when soybean trypsin inhibitor was added together with trypsin to microsomes showed that these proteins *per se* did not affect the kinase activity. When smooth microsomes were incubated with trypsin in the presence of Triton X-100, most of the enzyme activity disappeared showing that all the protein kinase fractions of microsomes are sensitive to proteolytic treatment.

To examine whether any of the protein kinase fractions was lost preferentially by the trypsin treatment DEAE-cellulose chromatography was performed on untreated and treated samples (after solubilisation in Triton X-100) (Fig. 5). Fraction MII seemed to be much more sensitive to trypsin than fractions MI and MIII, since its activity decreased from approximately 60% to 30% of the total activity recovered after each chromatography. This decrease has been confirmed in several experiments. Fraction MIII of the untreated samples resolved into two peaks on chromatography of which the first eluting peak disappeared on trypsin treatment. Since the recovery of protein kinase in both separations was around 100%, the approximate recovery of each enzyme fraction after trypsin treatment could be estimated. While fractions MI and MIII diminished to around 80% of their original activity, more than 70% of the activity of fraction MII disappeared. However, untreated fraction MIII consisted of two peaks, only one of which appeared to be digested. Therefore, the second peak of fraction MIII was most resistant to trypsin and fraction MII was most easily digested by the protease.

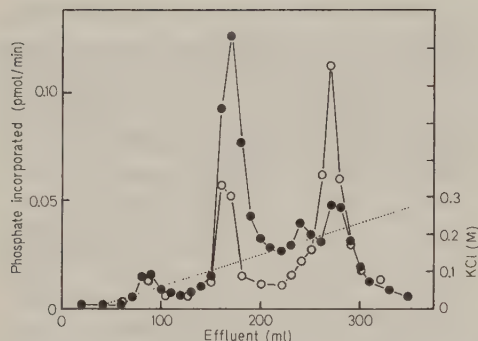


Fig. 5. DEAE-cellulose chromatography of protein kinases from smooth microsomes before and after trypsin treatment. Smooth microsomes were treated with trypsin as described in the text. The membranes were solubilised in 2% Triton X-100 and 0.25% Triton X-100 was present in the chromatography buffer. Protein kinase activity was measured in the presence of 5 μ M cyclic AMP. (●) Untreated microsomes (8.5 mg of membrane protein applied); (○) trypsin-treated microsomes (14 mg of protein applied); (·····) depicts of the KCl gradient

The samples used for these DEAE-cellulose runs were the same as those presented in Table 4. It can be seen in the table that approximately 80% of the protein kinase activity analysed in the absence of detergent disappeared upon trypsin treatment. Since the kinase substrate (protamine) probably can be phosphorylated only by enzymes situated close to or on the outer surface of the membrane and trypsinisation removed 70% of kinase fraction MII, we suggest that this kinase fraction is exposed on the outer surface of microsomal membranes. The other two microsomal protein kinase fractions which are less affected by trypsin may be buried in the membrane (at least the area around their catalytic site) and they are active with protamine as substrate only after the membrane structure has been disturbed by detergent.

Binding Studies between Protein Kinases and Smooth Microsomes

The incorporation of various isolated protein kinases to smooth microsomes was studied (Fig. 6). Of the cytosolic enzymes fraction CIIb bound 2–3 times more efficiently than fraction CIII, while fractions CIa and CIIa bound to a much smaller extent to the membrane even when added in a large excess (fraction CIIa). The amount of fractions CIIb and CIII bound increased with increasing amounts of enzyme added, and between 50–60% of the total added fraction CIIb and around 20% of fraction CIII sedimented with the membrane as compared to 1–3% of fraction CIIa. Triton X-100 stimulated the activity of bound protein kinase by approximately 50%, and

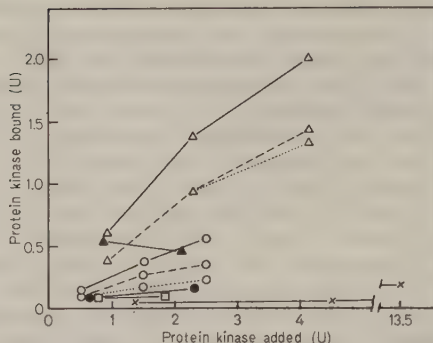


Fig. 6. Binding of protein kinase to smooth microsomes. Enzyme fractions were purified through the DEAE-cellulose chromatography step and incubated with smooth microsomes as described under Methods. The amount of protein kinase bound is plotted against the amount of enzyme added to an incubation mixture containing 0.59 mg membrane protein. One unit (U) of activity corresponds to 1 nmol of [32 P]phosphate incorporated into protamine per min at 22 °C. Enzyme analyses were performed in the absence of Triton X-100 with cyclic AMP (---) or without cyclic AMP (·····), and in the presence of 2% Triton X-100 with 5 μ M cyclic AMP (—). (□) Protein kinase fraction CIa; (×) CIIa; (Δ) CIIb; (○) CIII; (▲) MII and (●) MIII

the activity of fraction CIII, but not that of fraction CIIb, increased slightly by the addition of cyclic AMP.

The amount of enzyme bound was also quite substantial when compared to the basal protein kinase level in smooth microsomes. At the highest enzyme concentrations tested the membrane incorporated a 15-fold excess of fraction CIIb (7-fold when tested in the presence of Triton X-100) and a 4-fold excess of fraction CIII (2-fold in the presence of Triton X-100).

The microsomal enzyme fractions MII and MIII bound less efficiently to the membrane than corresponding cytosolic fractions (CIIb and CIII). The binding of fraction MII even diminished when its concentration was increased in the incubation mixture. It is possible that the less efficient binding of the microsomal kinase fractions was due to the presence of trace amounts of Triton X-100 derived from the solubilising medium and carried through the DEAE-cellulose chromatography step disturbing the interaction between enzymes and membrane.

DISCUSSION

The protein kinase fractions MI, MII and MIII found in microsomes appear to be identical with (or very similar to) the cytosol kinase fractions CIa, CIIb and CIII, respectively, as judged from their chromatographic and isoelectric focusing properties and from their substrate specificities. Since protein kinase fractions isolated from the cytosol also will combine with

smooth microsomes *in vitro* the question arises whether protein kinases become redistributed from cytosol to membrane during the preparation procedure, or whether the enzymes are distributed between these two fractions in the intact cell. There are several pieces of evidence in favour of the latter suggestion. Thus, 10% of the total protein kinase activity of rat liver cells (using protamine as substrate) is associated with the microsomes and thorough washing with 1.5 M KCl and sonication will remove only a minor portion of the enzyme from the membrane. In comparison, less than 0.1% of the total activity of a cytosol enzyme as lactate dehydrogenase is associated with the microsomes before the 1.5 M KCl wash. Detergents have to be used to solubilise the protein kinase and this enzyme is slightly more resistant to solubilisation than various microsomal marker components.

Another piece of evidence suggesting an intimate relationship between membrane and protein kinase is the latency of the activity of the membrane enzyme. An artifactual adsorption of the enzyme would most likely lead to at least a partial exposure of the catalytic site at the membrane surface and therefore, to a rather active enzyme. The trypsin digestion experiments suggest that at least one of the protein kinases associated with microsomes is quite inaccessible to an externally added substrate, since the latency of the remaining enzyme activity increased significantly upon digestion. The enzymes which associated with the membrane in the binding experiments were only activated slightly upon solubilisation of the membrane and might have been bound in a less specific manner than the original microsomal protein kinases.

A third argument for the existence of a specific binding between microsomal protein kinase and membrane is the fact that several microsomal proteins can undergo phosphorylation [6] (unpublished results). These phosphorylations can occur rapidly when both enzyme and substrate are present in rather large concentrations in the membrane phase where they can diffuse laterally. Furthermore, a compartmentalisation of phosphorylating enzymes would be a means to control the phosphorylation process, especially if a translocation mechanism for these enzymes exists as has been suggested [33, 34].

The latency of the activity of microsomal protein kinase shows that at least a fraction of the enzyme is not freely accessible on the membrane surface. The digestion experiments with trypsin suggest that fraction MIII is less accessible than fraction MII. On the other hand, fraction MIII is more susceptible to salt extraction than fraction MII. These results together with the detergent extraction data (Fig. 3) are compatible with a strong binding of fraction MII to the outer surface of microsomes, while it is less clear whether fraction MIII is buried in the membrane or maybe even exposed on the luminal membrane surface.

An interesting question is why no protein kinase corresponding to fraction CIIa is found in microsomes, and why fraction CIII but not fraction CIIa will bind to microsomes *in vitro*. It appears from our investigations that both these fractions as well as the microsomal protein kinase fraction MIII have the same two catalytic components. The binding would be expected, therefore, to be determined by regulatory components, either directly or through structural changes of the catalytic components induced by regulatory ones. Differences between the type I and type II protein kinases (most likely corresponding to our fractions CIIa and CIII, respectively) from various tissues due to their regulatory components have been reported. For instance, the type-II enzyme is less prone to dissociate in the presence of histones or salt than the type-I enzyme [26] and its regulatory component may undergo a rapid autophosphorylation [35, 36]. A large part of the type-II enzyme of heart tissue but not the type-I enzyme will sediment with particulate material [37], results in line with our findings for rat liver microsomal enzymes. The mechanism of binding of the enzymes to respective particulate material appears to differ, however. The heart enzyme can be solubilised by trypsin treatment and no latency of its activity is apparent upon solubilisation with Triton X-100 [37] which is in contrast to our results concerning the microsomal enzyme. We are presently examining the binding mechanism between protein kinases and microsomal membranes, and especially the possible importance of regulatory components.

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Protein Kinase Activity and Endogenous Protein Phosphorylation in Rat Liver Plasma Membranes

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When rat liver plasma membranes were incubated with [γ - ^{32}P]ATP radio-labelled phosphate was incorporated into endogenous protein and exogenous substrate by a membrane-bound protein kinase activity. A high ATP/membrane protein ratio was required for optimum incorporation conditions. Cyclic AMP did not affect the incorporation of phosphate. The protein kinase activity was extracted from the membranes by 1 % Triton X-100 and a high concentration of KCl. The solubilised enzyme resolved into two fractions on DEAE-cellulose chromatography. One enzyme fraction had the same properties as the catalytic subunit of cytosolic cyclic AMP-dependent protein kinases. Endogenously phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis into five major and additional minor phosphorylated components. Three of the major phosphorylated components were tightly bound to the membrane material and were not extracted by 1 % Triton X-100 and 1M KCl.

The activity of several proteins is regulated by enzymically catalysed phosphorylation-dephosphorylation reactions.¹ Protein phosphorylation in rat liver is a feature of most subcellular fractions,^{2–4} and protein kinases have been isolated and characterised from the cytosol,^{3,5,6} microsomes,³ mitochondria^{7–9} and nuclei.^{10,11} Comparatively little is known about protein kinases and the phosphorylation of proteins in rat liver plasma membranes. Early studies by Blat and Harel¹² showed that [^{32}P]phosphate was incorporated into proteins of such membranes *in vivo*, and that isolated plasma membranes catalysed the incorporation of phosphate from [γ - ^{32}P]ATP into endogenous proteins.¹³ Apparently, partially purified protein kinase from rat liver cytosol also catalyses the phosphorylation of plasma membrane proteins.¹⁴

We have previously examined protein phosphorylation, protein kinases and phosphoproteins in rat liver membrane fractions including microsomes and mitochondria.^{3,4,9} Our investigations have now been extended to plasma membranes.

EXPERIMENTAL

Chemicals. [^{32}P]-Inorganic phosphate was obtained from the Radiochemical Centre (Amersham). [γ - ^{32}P]ATP was synthesised according to Chang *et al.*¹³ Protamine sulfate, calf thymus histones (Type II-A) and rabbit muscle protein kinase inhibitor were from Sigma, adenosine deaminase from Boehringer, acrylamide from Bio-Rad, and *N,N'*-methylene bisacrylamide (which was recrystallised twice from acetone) from Eastman. All other chemicals were of reagent grade.

Preparation of plasma membranes. Male Sprague-Dawley rats fed *ad libitum* were stunned by a blow on the head and bled by cutting the carotic artery. Livers were excised immediately and chilled at 0 °C in 5 mM Tris-HCl, pH 8.0, 0.25 M sucrose and 0.1 mM dithioerythritol. All subsequent preparations were at 0–4 °C.

Plasma membranes were prepared according to Aronson and Touster.¹⁶ The N_2 fraction, which was less contaminated by other membranes than the P_2 fraction as based on marker enzyme analyses, was used. The average relative specific activities of marker enzymes in several preparations of the N_2 fraction compared to the tissue homogenate were 34 for 5'-nucleotidase (measured by method 3 in Ref. 17); 0.3 for lactate dehydrogenase;¹⁸ 0.03 for cytochrome oxidase (measured as described in Ref. 19 but using an extinction coefficient of $21 \text{ mM}^{-1} \text{ cm}^{-1}$);²⁰ 1.0 for NADPH-cytochrome *c* reductase;²⁰ and 6.0 for *N*-acetylglucosamine galactosyltransferase.²¹

Analyses. Protein kinase activity and endogenous phosphorylation were assayed as described earlier^{22,23} with the following modifications. The protein kinase assay mixture contained 2 mM [γ -³²P]ATP (50 000–100 000 dpm/nmol) and 3 μ g of membrane protein in a final volume of 50 μ l unless otherwise stated, and the incubation time was 1 min at 25 °C. Endogenous phosphorylation was determined in a medium containing 2 mM [γ -³²P]ATP (50 000–100 000 dpm/nmol), 20 mM sodium azide and 20 μ g of protein in a final volume of 50 μ l. The incubation time was 5 min at 25 °C. In the time course experiments the reaction mixture was scaled-up and samples (50 μ l) were withdrawn at indicated times.

Substrate specificity was examined using protamine or a histone mixture as substrate.²³ Protamine phosphorylation was examined in the presence and absence of 0.3 M NaCl, and the phosphorylation of histones in the absence of salt. The amount of histone was 0.6 mg per 100 μ l of incubation mixture.

The inhibition of protein kinase activity by protein kinase inhibitor²⁴ was studied using a fixed amount of enzyme and increasing the concentrations of the inhibitor. The activity was assayed in the presence of 10 μ M cyclic AMP using protamine as a substrate.

Isoelectric focusing was as described previously.³ Protein was measured by the Lowry method²⁵ after precipitation with 10 % trichloroacetic acid. Bovine serum albumin was used as a standard. All determinations were in duplicate.

Extraction of protein kinase from plasma membranes. For extraction experiments plasma membranes were incubated with 0.3 or 1 M KCl in the presence or absence of 1 % Triton X-100. After incubation for 30 min at 0 °C samples were centrifuged at 105 000 $\times g$ for 90 min. The supernatants and pellets resuspended in buffer (10 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 0.1 mM dithioerythritol) were analysed for protein kinase, endogenous incorporation of phosphate, 5'-nucleotidase and protein content.

DEAE-cellulose chromatography. Plasma membranes were extracted in 1 % Triton X-100 and 1.0 M KCl as described above. The extract was dialysed extensively against 10 mM Tris-HCl, pH 7.4, 1 mM MgCl₂ and 0.1 mM dithioerythritol. The dialysed extract was applied onto a DEAE-cellulose (Whatman DE-52) column and the chromatography was developed with a linear KCl gradient.³ The eluate was analysed for protein kinase activity using 0.5 mM [γ -³²P]ATP and protamine sulfate as a substrate.²⁶

SDS-polyacrylamide gel electrophoresis. Endogenous phosphorylation for electrophoresis was with 20–100 μ g of membrane protein as described

above using [γ -³²P]ATP with a specific activity of 0.8×10^6 dpm/nmol. Electrophoresis was according to Neville²⁶ with some modifications.⁹ The further treatment of the gels and autoradiography have been described previously.⁹ The following standard proteins were used: bovine serum albumin, M_r 69 000; ovalbumin, M_r 45 000; soybean trypsin inhibitor, M_r 21 500; and sperm whale myoglobin, M_r 17 200.

RESULTS

Time course of protein kinase activity and endogenous phosphorylation. In initial experiments we found that purified plasma membranes from rat liver catalysed the incorporation of phosphate from ATP into protamine and that there was also incorporation into endogenous substrates. These experiments further indicated that a much higher ATP/membrane protein ratio was required in plasma membranes than in other membranes from rat liver cells to obtain optimum conditions for the incorporation of phosphate into both exogenous and endogenous substrates.

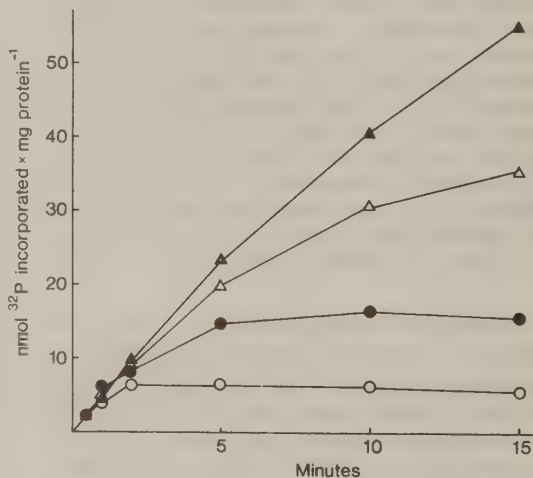


Fig. 1. Time course of plasma membrane protein kinase activity. Plasma membranes (3 or 20 μ g of membrane protein per 50 μ l incubation mixture) were incubated with protamine as exogenous substrate as described under Experimental, and the incorporation into trichloroacetic acid-sodium tungstate precipitable phosphate was determined. Analyses were performed in presence (closed symbols) or absence (open symbols) of 20 mM sodium azide. $\triangle$, 3 μ g of membrane protein; $\circ$, 20 μ g of membrane protein.

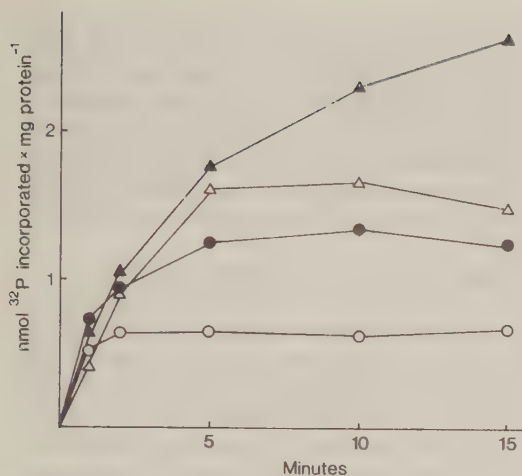


Fig. 2. Time course of endogenous incorporation of phosphate into plasma membranes. Incubations were performed as described under Experimental, and the incorporation of phosphate into trichloroacetic acid – sodium tungstate precipitable material was determined. Symbols represent the same amounts of plasma membranes and sodium azide as in Fig. 1.

The time courses of plasma membrane protein kinase activity obtained with various amounts of membrane protein and 2 mM ATP are presented in Fig. 1. The effect of the ATPase inhibitor sodium azide was also tested. The rate of incorporation was linear for 2 min at a low membrane protein concentration (3 μg per 50 μl incubation medium) and then decreased slowly. At 20 μg the incorporation levelled off already after 2 min. This was probably due to exhaustion of ATP caused primarily by membrane ATPase, since the reaction proceeded for a longer time in the presence of azide. In comparison, when the incubation conditions used earlier for microsomes and mitochondria^{3,9} were tested with plasma membranes the incorporation ceased within 20 s.

The endogenous incorporation of phosphate into plasma membranes followed similar time courses as the protein kinase activity (Fig. 2), although the initial reaction rate was 10 times lower than with exogenous substrate. At the low membrane concentration, maximum incorporation was reached after 5 min with 1.6 nmol of phosphate incorporated per mg of membrane protein. Further incorporation was obtained in the presence of azide. Cyclic

Table 1. Extraction of plasma membrane components with KCl and Triton X-100. Purified plasma membranes were treated with KCl or KCl and Triton X-100 as described in the experimental section. Analyses were performed on extracts and unextracted material (pellets) and the distribution of recovered activities was calculated.

Treatment	Protein kinase activity (nmol min ⁻¹)		Endogenous incorporation of phosphate (nmol)		Protein (mg)		5'-Nucleotidase ($\mu\text{mol min}^{-1}$)	
	Extract	Pellet	Distribution extract-pellet (%)	Extract	Pellet	Distribution extract-pellet (%)	Extract	Pellet
Membrane suspension	3.63			1.55	2.10		0.97	
0.3 M KCl	0.23	5.39	4-96	0.11	0.20	8-92	0	0.39
1.0 M KCl	0.81	4.33	16-84	0.06	0.22	6-94	0.004	0.35
0.3 M KCl + 1 % Triton X-100	1.92	1.30	60-40	0.66	1.22	69-31	0.065	0.31
1.0 M KCl + 1 % Triton X-100	2.50	0.35	88-12	0.54	1.25	67-33	0.46	0.19

AMP did not stimulate the phosphorylation of either endogenous or exogenous substrates.

Extraction of protein kinase from plasma membranes. To examine how tightly the protein kinase activity was attached to the membranes, extraction experiments were performed. Isolated plasma membranes were treated with various KCl and Triton X-100 concentrations and the distribution of protein kinase, endogenous phosphorylation, protein and the plasma membrane marker enzyme 5'-nucleotidase between solubilised and residual material was measured (Table 1). No 5'-nucleotidase activity and only a small fraction of protein kinase, endogenously phosphorylated material and protein was released from the plasma membranes on treatment with 0.3 M or 1 M KCl. However, a major part of protein kinase activity, endogenously phosphorylated material and protein was extracted in the presence of both salt and Triton X-100. The bulk of the protein appeared to be extracted under less drastic conditions than the protein kinase, since the protein was released at lower concentrations of Triton X-100 than most of the protein kinase activity (not shown). Most of the 5'-nucleotidase activity remained in the particulate fraction at 0.3 M KCl and 1 % Triton X-100, but was released when the salt concentration was increased to 1 M in presence of the detergent.

The protein kinase activity of plasma membranes increased by approximately 50 % on treatment

with KCl. The recovery of protein kinase after detergent treatment was lower, however, than the activity present in the parent membrane suspension. This is in contrast to what we have found earlier in microsomes and mitochondria where the protein kinase activity was latent in the membrane and increased several-fold in the presence of Triton X-100.^{3,9}

DEAE-cellulose chromatography. Plasma membranes were treated with 1 % Triton X-100 and 1 M KCl, and the solubilised material was subjected to DEAE-cellulose chromatography. Two peaks (PM I and PM II) with protein kinase activity were resolved (Fig. 3). When eluted from the column none of these peaks was stimulated by cyclic AMP. The two peaks eluted at the same salt concentrations (0.03 and 0.20 M, respectively) as two (M I and M III) of the three protein kinase peaks from rat liver microsomes³ and rat liver mitochondria.⁹ Two protein kinases that elute under these conditions have also been found in rat liver cytosol, which, in addition, contains two other enzyme peaks.³ Occasionally a third plasma membrane protein kinase fraction was observed which eluted at the same KCl concentration (0.14 M) as the M II fraction of microsomes and mitochondria.

Characterisation of the protein kinase fractions. Analyses of the protein kinase peaks from microsomes and the cytosol³ have shown that the first eluting peak is the catalytic subunit of the cyclic

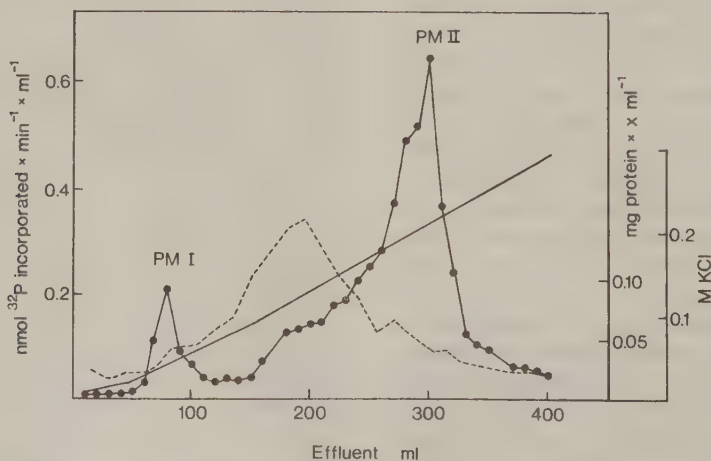


Fig. 3. DEAE-cellulose chromatography of plasma membrane protein kinase activity. Conditions used were as described in the experimental section. Protein kinase activity (●) was determined with protamine as substrate. ---, protein concentration measured according to Lowry; —, KCl gradient. The applied amount of protein was 15 mg.

Table 2. Substrate specificity of protein kinases and activity in presence of protein kinase inhibitor. Protein kinases were isolated from plasma membranes and cytosol by DEAE-cellulose chromatography. Their substrate specificities were determined in presence of 5 μ M cyclic AMP. The designations of the cytosolic protein kinases are the same as in Ref. 3. Activities are expressed relative to the activity towards protamine in presence of 0.3 M NaCl. The activity with protein kinase inhibitor was determined under conditions of maximum inhibition with protamine as substrate.

Substrate	Plasma membrane protein kinase		Cytosolic protein kinase			
	PM I	PM II	C I	C IIa	C IIb	C III
Protamine + NaCl	100	100	100	100	100	100
Protamine - NaCl	42	106	41	41	90	77
Histone mixture	18	9	7	9	4	12
Activity with protein kinase inhibitor	14	70	24	27	97	52

AMP dependent protein kinases, the second peak is a cyclic AMP independent enzyme while the third peak is the cyclic AMP dependent type II²⁷ protein kinase. Isoelectric focusing of PM I showed that this peak had the same isoelectric point ($pI=8.1$) as the catalytic subunit isolated from the cytosol.³ Although the PM II fraction was unstable on isoelectric focusing (the recovery was 2 % compared to 25–40 % obtained for other protein kinases examined in this manner) two protein kinase peaks could be observed with the isoelectric points 5.5 and 4.9. These correspond to the pI values we obtained earlier with the cyclic AMP-independent protein kinase and the cyclic AMP-dependent type II enzyme, respectively.³

The PM I and PM II protein kinase fractions were characterised further by their substrate specificity (of particular interest was the influence of NaCl on the phosphorylation of protamine) and the effect of a protein kinase inhibitor isolated from rabbit skeletal muscle (Table 2). Both substrate specificity and maximum inhibition by the protein kinase inhibitor were similar for PM I and the cytosolic protein kinase fraction C I (for designations of cytosolic kinases, see Ref. 3), providing further evidence that PM I is the catalytic subunit of cyclic AMP-dependent protein kinases. The activity of the PM II fraction decreased 30 % in the presence of inhibitor, and the rate of protamine phosphorylation by this enzyme was independent of the concentration of NaCl. In comparison, the decrease in activity of the cyclic AMP-dependent cytosolic protein kinases C IIa and C III due to the presence of inhibitor was 73 and 48 %, respectively, while the cyclic AMP-independent enzyme C IIb

was unaffected. The lower inhibition of fraction C III compared to C IIa was probably due to an incomplete dissociation of the holoenzyme under the experimental conditions used. The phosphorylation of protamine catalysed by fraction C IIb was also unaffected by 0.3 M NaCl, while the usual stimulation by salt was observed for the cyclic AMP-dependent enzyme fractions. The rate of histone phosphorylation was similar for the different enzyme fractions.

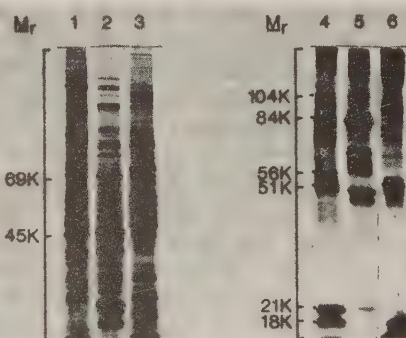


Fig. 4. Protein and phosphoprotein patterns of plasma membranes (lane 1 and 4), cytosol (2 and 5) and smooth microsomes (3 and 6) from rat liver. 1–3, protein patterns; 4–6, phosphoprotein patterns obtained by autoradiography. Standard proteins are indicated to the left of lane 1 and the apparent M_r of phosphorylated components to the left of lane 4. The protein patterns are from a different run than the phosphoprotein patterns. Smooth microsomes and cytosol were prepared as previously described.³

Phosphoprotein and protein patterns. To examine phosphoprotein and protein patterns, plasma membranes were phosphorylated endogenously using [γ - 32 P]ATP followed by SDS-polyacrylamide gel electrophoresis and autoradiography. For a comparison we also examined smooth endoplasmic reticulum and cytosol from rat liver in the same way. The protein patterns of plasma membranes, smooth microsomes and cytosol were distinctly different (Fig. 4), indicating that plasma membranes were not seriously contaminated by the latter two fractions. The phosphoprotein patterns were also different. Five prominent phosphorylated components (labelled 84K, 56K, 51K, 21K, and 18K in the figure after their apparent M_r) and additional minor ones were resolved from plasma membranes. Three of the major components seemed to be unique to plasma membranes while one (21K) had the same mobility as a phosphorylated component in the cytosol and another (18K) the same as one of the microsomal components. The 18K and 21K components remain unidentified so far, and it is not known whether they represent proteins present in more than one subcellular fraction, or whether they fortuitously have the same mobility as unrelated phosphorylated proteins in microsomes or the cytosol.

The phosphoprotein pattern of plasma membranes was also examined after various incubation times (not shown). The strength of all radioactive bands increased similarly when the incorporation of phosphate increased indicating a simultaneous phosphorylation of the components under the experimental conditions used.

The phosphoprotein patterns obtained after different extractions are shown in Fig. 5. Four prominent phosphorylated components (59K, 49K, 41K, 39K) were seen in the 1M KCl-extract. Of these the 59K, 49K and 39K components were not seen in the parent membrane, while the 41K component was a minor phosphorylated band in the native membrane which became heavily phosphorylated after extraction. Additional phosphoprotein bands (104K and 51K) were seen in the KCl and Triton X-100 extract. Interestingly, the 56K, 51K (in part) and 21K components remained in the particulate fraction after this drastic treatment which removed 80 % of the membrane proteins, while the 18K component was found neither in the extract nor in the pellet.

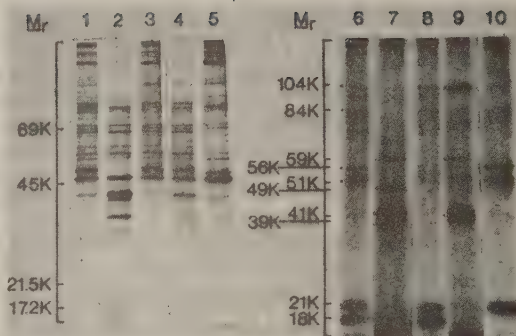


Fig. 5. Protein and phosphoprotein patterns of plasma membranes after various extractions. Extractions were performed with 1M KCl or 1% Triton X-100 and 1M KCl as described in the experimental section. Extracts and residues were phosphorylated endogenously before electrophoresis and autoradiography. 1-5 are protein patterns and 6-10 are phosphoprotein patterns in the order plasma membranes, KCl extract, residue after KCl extraction, Triton X-100 + KCl extract, residue after Triton X-100 + KCl extraction. Standard proteins are indicated to the left of lane 1 and the apparent M_r of phosphorylated components to the left of lane 6.

DISCUSSION

Several pieces of evidence indicate that the isolated protein kinases and phosphoproteins are harboured in the plasma membrane rather than representing contamination from other subcellular fractions. First, plasma membranes were substantially enriched during the preparation compared to other subcellular fractions (for instance, they were enriched 35-fold as compared to microsomal membranes). Second, both protein and phosphoprotein patterns were unique to the plasma membrane fraction. Third, no latency of protein kinase activity was observed when plasma membrane were treated with Triton X-100. In comparison, there is a several-fold increase in the kinase activity upon treatment of microsomes,³ mitochondria⁹ or Golgi membranes (unpublished) with the detergent. Furthermore, the protein kinases appeared to be tightly bound to the membrane, since a rather harsh treatment with detergent and a high salt concentration were required to solubilise the enzyme activity.

Cyclic AMP did not stimulate the membrane-associated or solubilised kinase when analysed with

either endogenous or exogenous substrates, contrary to what has been found for plasma membrane protein kinases from other kinds of cells.^{28,29} We observed, however, that cyclic AMP-binding material is present in liver plasma membranes (approximately 2 pmol cyclic AMP bound per mg of membrane protein). This material has not been further characterised and it is unclear whether it represents any of the regulatory subunits of cyclic AMP-dependent protein kinases or other cyclic AMP-binding components.

The specific activity of the protein kinase of plasma membranes was several-fold higher than in microsomes³ or mitochondria.⁹ However, the enzyme activity of plasma membranes did not show any latency on detergent treatment in contrast to the activity in microsomes or the mitoplast fraction of mitochondria. The lack of latency indicates that the substrate (protamine) is freely accessible to the protein kinase. Since we have found in preliminary experiments that only a few proteins in isolated plasma membranes become significantly labelled by diazotised iodosulfanilic acid, all membranes probably expose the same surface to the surrounding medium. The protein kinase may therefore be attached to only one plasma membrane surface in intact cells. This is likely to be the outer surface.^{30,31} It has been reported that protein kinases are exposed on the outer surface of various kinds of cells including adipocytes³² and Ehrlich ascites tumour cells.³³

The protein kinase of plasma membranes was resolved into two enzymically active fractions on DEAE-cellulose chromatography. The first eluting fraction (PM I) is likely to represent the catalytic subunit of cyclic AMP-dependent protein kinases based on its isoelectric point, its substrate specificity and its inhibition by the protein kinase inhibitor. We have found earlier that the catalytic subunit apart from being present in the cytosol is also present in small amounts in rat liver microsomes³ and mitochondria.⁹ The subunit therefore seems to be widely distributed in the cell. The possibility that it is a contaminant from the cytosol appears less likely since a cytosolic enzyme as lactate dehydrogenase was present in a very low concentration in purified plasma membranes and since drastic conditions were required to solubilise protein kinase from the membrane.

The identity of the second kinase fraction (PM II) is less clear. It eluted from DEAE-cellulose at the same KCl concentration as the cyclic AMP-

dependent type II protein kinase which also seems to be present in microsomes and mitochondria.^{3,9} The PM II fraction was partly inhibited by the protein kinase inhibitor, but contrary to what is expected for cyclic AMP-dependent protein kinases,³ NaCl did not activate the phosphorylation of protamine by this fraction. Furthermore, the enzyme activity was unaffected by cyclic AMP when eluted from the column. However, a stimulation by this nucleotide is not necessarily expected with such a dilute enzyme as in this case (*cf.* Ref. 9). Due to the lability of the enzyme we have not yet been able to perform detailed studies of its properties.

Only a few proteins became heavily phosphorylated on endogenous phosphorylation of isolated plasma membranes. Most of these phosphoproteins were tightly bound to the particulate material since they were not released by treatment of the membrane with detergent and high KCl concentrations. Some proteins which were not phosphorylated significantly in the isolated membrane were amenable to phosphorylation after treatment with KCl and/or detergent. It is possible therefore that there are restrictions in enzyme-substrate interactions in the isolated membrane, either due to different transverse locations of enzymes and substrates, or to restrictions in their lateral mobility. If some protein substrates are located on the inner surface of intact cells while protein kinases are located on the outside, these substrates may be available for phosphorylation only by cytosolic protein kinases. We are presently studying the transverse location of protein kinases and their substrates in plasma membranes to resolve these questions.

As to the biochemical significance of protein phosphorylation in rat liver plasma membranes, one can only speculate as long as the phosphoproteins remain unidentified. Since protein phosphorylation in general seems to be connected with various regulatory functions in the cell, it is likely that the phosphorylation of proteins in plasma membranes also is a regulatory device. Thus, it has been suggested that the state of protein phosphorylation in erythrocyte membranes may regulate the membrane structure,³⁴ and there is indirect evidence for a correlation between protein phosphorylation and the movement of Na⁺ ions through the erythrocyte membrane.³⁵ There is also a recent report³⁶ on protein kinase-catalysed phosphorylation of the Na⁺, K⁺-ATPase of kidney plasma membranes.

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CYCLIC AMP-DEPENDENT PROTEIN PHOSPHORYLATION ON THE SURFACE OF RAT HEPATOCYTES

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1. Introduction

Proteins of isolated plasma membranes from rat liver cells can be phosphorylated either by endogenous [1–3] or exogenously added [4] protein kinases. Protein phosphorylation in plasma membranes presumably serves as a general regulatory mechanism as is the case in other subcellular fractions. An example is the phosphorylation and thereby activation of a cyclic nucleotide phosphodiesterase associated as a peripheral protein with rat liver plasma membranes [5]. The phosphorylation of other, as yet unidentified, plasma membrane proteins appears to be modulated by insulin [3].

No studies have been performed so far on the phosphorylation of proteins on the external surface of isolated rat hepatocytes. The phosphorylation of surface proteins in other kinds of cells has been described, however [6–8]. An examination of surface protein phosphorylation may yield information about regulatory mechanisms on the plasma membrane level. Here we report on cyclic AMP-dependent and independent phosphorylation of proteins on the outer surface of rat hepatocytes.

2. Experimental

Hepatocytes were prepared [9,10] from male Sprague-Dawley rats weighing 200–300 g. Bicarbonate-free Hanks' medium [11], buffered to pH 7.4 with 20 mM HEPES, also containing 0.05% collagenase, 0.1% hyaluronidase and 0.2% bovine serum albumin (all from Sigma) was used for the perfusion. The viability of the cells, measured as exclusion of 0.1% trypan blue, was above 90%.

In control experiments to examine the influence of leakage of protein kinase and cyclic adenosine 3', 5'-monophosphate (cyclic AMP) binding proteins on hepatocyte surface phosphorylation 1 g of hydroxyapatite-agarose gel (HA-Ultrogel, LKB) was added per g liver, when the perfused and collagenase-treated liver was dispersed in medium. The gel was present during the entire handling of the cells before phosphorylation analyses. The recovery of cells dropped to approx. 50% by this treatment due to adsorption of cells to the gel, but the viability of the remaining cells increased to above 95%.

Protein kinase analyses were performed (usually in triplicate) at 37°C in Ca²⁺-free Hanks' medium buffered with 20 mM HEPES, pH 7.4. 1 ml of incubation mixture contained 2 µmol [γ -³²P]ATP (100–150 dpm/pmol; prepared as in [12]), 3 mg histones (Sigma, type II A), 10–15 × 10⁶ hepatocytes, and (when indicated) 5 nmol cyclic AMP. At indicated times 50 µl of the mixture was spotted on a Whatman 3 MM filter paper disc, which was washed and counted as in [13]. Protein phosphatase activity was followed after the addition of hexokinase (Sigma, 180 U/ml) to remove unreacted ATP (glucose is present in the incubation medium). In some experiments 360 µg/ml rabbit muscle protein kinase inhibitor (Sigma) was added.

Endogenous phosphorylation of hepatocytes was performed as above but omitting histones. The filter paper discs were immersed immediately in 10% trichloroacetic acid. Before counting they were washed in the following way to remove unreacted ATP and radioactively labelled phospholipids (particularly phosphoinositides, cf. [14]): twice for 10 min in 10% trichloroacetic acid and 1 M H₃PO₄, boiled once for 15 min in 5% trichloroacetic acid, washed once each for 5 min in 10% trichloroacetic acid and 1 M H₃PO₄,

ethanol, ethanol/ether (1:1, by vol.), chloroform/methanol 12 M HCl (200:100:1, by vol.), and ether. The discs were dried and counted in 0.4% Omnifluor (New England Nuclear) in toluene in a liquid scintillation counter.

Samples for electrophoresis were incubated for 5 min in 50 μ l medium also containing 0.1 μ mol [γ - 32 P]ATP (800–1000 dpm/pmol), 200 000 hepatocytes, and 0.25 nmol cyclic AMP (when indicated). SDS-polyacrylamide gel electrophoresis and autoradiography of the gels were as in [15,16].

3. Results

3.1. Phosphorylation of exogenous substrate

To test whether hepatocytes possess protein kinase activity on their external surface, isolated cells were incubated with [γ - 32 P]ATP and histones as exogenous substrate (fig.1). There was an incorporation of radioactively labelled phosphate, and cyclic AMP stimulated the reaction by approx. 50%. The cyclic AMP-dependent incorporation was partly inhibited by rabbit muscle protein kinase inhibitor [17], which acts specifically on cyclic AMP-dependent protein kinases. When hexokinase was added to remove unreacted ATP, a time-dependent decrease of bound phosphate was observed (----). This indicates the presence of phosphoprotein phosphatase activity as well on the cell surface.

The rate of phosphate incorporation was fast initially, and then slower and close to linear for a few

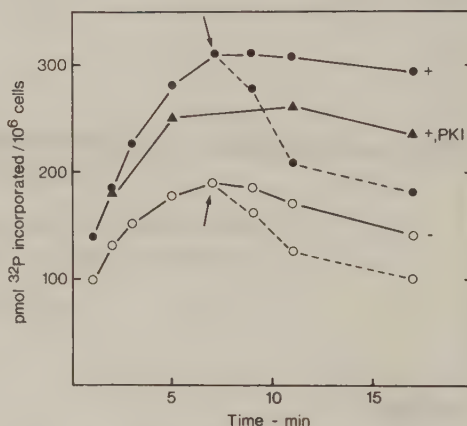


Fig.1. Phosphorylation and dephosphorylation of histones by hepatocytes. Analyses were performed as in section 2 in presence (+) or absence (–) of cyclic AMP. Arrows indicate addition of hexokinase to follow the dephosphorylation (----). PKI, protein kinase inhibitor.

minutes. The rate of incorporation was then approx. 40 and 25 pmol of phosphate/ 10^6 cells per min in presence and absence of cyclic AMP, respectively. The rate of dephosphorylation was also higher in presence of cyclic AMP, presumably due to a higher concentration of phosphate groups on the substrate in this case as compared to incubations without cyclic AMP.

The possibility that the protein kinase activity was due to an association of intracellular enzymes released

Table 1
Effect of adsorption of released protein kinase on hepatocyte surface phosphorylation

Treatment during cell preparation	Protein kinase in cell washes (nmol/min per g liver)		Cyclic AMP binding in cell washes (pmol/g liver)	Hepatocyte protein kinase (pmol/min per 10^6 cells)		Endogenous phosphorylation (pmol/min per 10^6 cells)	
	– ^a	+ ^a		–	+	–	+
Untreated	4.36	6.22	6.23	23.1	30.8	2.87	3.62
Hydroxyapatite agarose gel	1.12	1.21	1.34	36.0	44.1	3.52	3.95

After dispersion of the collagenase-treated liver in the absence or presence of hydroxyapatite the cells were incubated for 15 min at 37°C and washed twice as in [10]. The combined supernatants were analyzed for protein kinase (see section 2) and cyclic AMP binding (by a slight modification of [20]), and the cells for protein kinase and endogenous phosphorylation

^a – and +, in the absence or presence of cyclic amp, respectively

from leaking or broken cells during the preparation was considered. Approx. 80% of the released protein kinase and cyclic AMP-binding proteins were adsorbed by including hydroxyapatite in the cell preparation medium (table 1). The concentration of protein kinase on the hepatocyte surface did not decrease by this treatment (it rather increased in this particular, but not in other, control experiments), which would have been expected if released enzyme components associate with the cell surface. The hepatocytes could also be incubated with liver cytosol without an increase in the surface protein kinase activity (not shown).

3.2. Endogenous phosphorylation

The incorporation of phosphate into endogenous substrates was examined next (fig.2). The rate of this reaction was approx. 15 times lower than with histones as substrates (3 and 2 pmol incorporated/ 10^6 cells per min in presence and absence of cyclic AMP, respectively). A dephosphorylation of endogenously phosphorylated material occurred after addition of hexokinase to remove unreacted ATP. The rate and extent of this dephosphorylation was less than that observed with exogenous substrate. The endogenous phosphorylation was not affected when cells were prepared in the presence of hydroxyapatite to adsorb material released from leaking or broken cells (table 1).

The protein kinase inhibitor from rabbit skeletal muscle inhibited endogenous phosphate incorporation (fig.2). The rate of incorporation in presence of both inhibitor and cyclic AMP was similar to that obtained

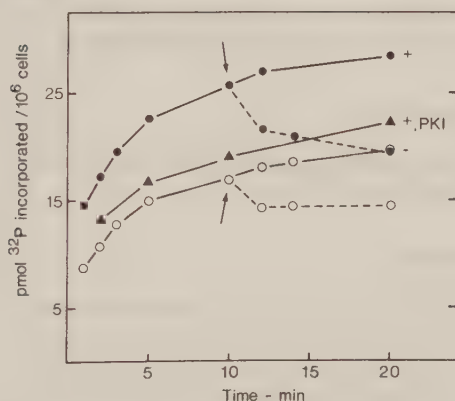


Fig.2. Endogenous phosphorylation and dephosphorylation of hepatocyte surface proteins. See legend to fig.1.

Table 2
Subcellular location of phosphorylated components

Preparation	Total incorporation	Supernatant	Pellet	% recovery
Intact cells	21.9	0.1	9.9	46
Homogenized cells	73.2	6.8	35.6	58

Hepatocytes or homogenized cells (30 strokes of a tight-fitting Dounce homogenizer) were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 5 min at 37°C . A 10-fold excess of unlabelled ATP and 0.05 M sodium fluoride were added at 0°C to stop further labelling and to inhibit phosphatase activity. The cells were homogenized as above and centrifuged at $110\,000 \times g$ for 90 min. Samples from supernatants and pellets were spotted on filter paper discs and washed as for endogenous phosphorylation before counting. The incorporation is given as pmol/ 10^6 cells

without these additions. The inhibitor did not affect the cyclic AMP-independent incorporation appreciably (not shown).

To verify that the endogenous phosphorylation occurred on the hepatocyte surface, and was not due to leakage of radioactively labelled ATP into the cells or of intracellular material to the medium, we examined the incorporation of phosphate into soluble and particulate fractions of hepatocytes (table 2). The radioactivity was recovered almost exclusively in the particulate fraction after incubation of intact cells with radioactively labelled ATP. If, however, the cells were homogenized before the incubation, an appreciable incorporation was into the soluble fraction, i.e. cytosolic material. The incorporation into particulate material was also higher in homogenized cells, presumably due to the phosphorylation of components in the exposed intracellular membranes. These findings strongly indicate that the incorporation into intact cells was localized to the cell surface.

3.3. Phosphoprotein patterns

Phosphoprotein patterns were examined by SDS-polyacrylamide gel electrophoresis and autoradiography after incubation of hepatocytes with radioactively labelled ATP (fig.3). A few proteins became labelled, and the incorporation into 5 of these (indicated by dots) was dependent on cyclic AMP. Presumably the incorporation was into surface proteins, since the pattern did not change by including hydroxy-

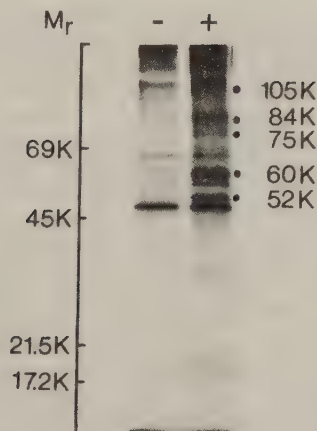


Fig.3. Phosphoprotein patterns. Hepatocytes were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in presence (+) or absence (-) of cyclic AMP. Proteins were separated by SDS-polyacrylamide gel electrophoresis and autoradiographed. Dots indicate components with a cyclic AMP-stimulated phosphorylation. The M_r of reference proteins is indicated to the left.

apatite in the cell preparation medium, and since no $[\text{P}^{32}]\text{phosphate}$ was found in the soluble fraction after homogenization of the cells (see above). We have also found in other experiments that the phosphoprotein patterns obtained with homogenized cells or with rat liver cytosol are quite different from the patterns presented here.

4. Discussion

The results provide evidence for a surface location of protein kinase, phosphoprotein phosphatase, and substrates for these enzymes in isolated rat liver hepatocytes. At least a part of the exposed protein kinase activity is cyclic AMP dependent. The evidence for a surface location is based on the phosphorylation/dephosphorylation of histones (a substrate which presumably does not enter intact cells) and the incorporation of phosphate into particulate, but not into soluble, material in the cell. In addition, there was no lag phase in the phosphorylation of endogenous proteins. This would have been expected if labelled ATP or phosphate had to enter the cells before phosphorylation, since these would have to equilibrate with

intracellular pools to yield a maximum rate of incorporation. Thus, a lag phase of at least 10 min is seen in the phosphorylation of proteins when hepatocytes are incubated with $[\text{P}^{32}]\text{phosphate}$ (unpublished).

The possibility that intracellular enzymes catalyzed the cell surface phosphorylation after leakage to the medium and association with the plasma membrane appears less likely, since 80% of the protein kinase and cyclic AMP-binding proteins set free during the cell preparation could be trapped without decreasing the protein kinase activity of the cells. The possible leakage of enzymes during collagenase treatment could not be checked, however. Since the liver was continuously perfused during this stage released enzymes were likely to be carried away before they could associate extensively with the cell surface.

Cyclic AMP-dependent protein kinases at low concentrations do not bind extensively to microsomal membranes [18], and we did not observe any increase in the protein kinase activity of hepatocytes after incubation of the cells with liver cytosol. The leakage of protein kinase from the cells during analysis was also low.

The functional importance of a location of cyclic AMP-dependent protein kinase and substrates for this enzyme on the hepatocyte surface is a matter for speculation. It is in line, however, with the recent finding [19], that a fraction of the cellular cyclic AMP synthesis may take place on the outer surface of hepatocytes. Since a surface location of cyclic AMP-dependent protein kinase activity has been reported for several kinds of cells [7,8], this might be a property of most cells. It is of interest now to identify the phosphoproteins located on the hepatocyte surface, and to examine the function of these phosphorylations.

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Ca²⁺- AND PHOSPHOLIPID-DEPENDENT PROTEIN KINASE IN RAT LIVER CYTOSOL

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SUMMARY

A Ca^{2+} - and phospholipid-dependent protein kinase from rat liver cytosol has been purified 300-fold by chromatography on DEAE-Sephacel and hydroxylapatite. The addition of Ca^{2+} and phospholipids was necessary for full enzyme activity when histone H1 was the substrate, while the phosphorylation of protamine was inhibited by these substances. Of the phospholipids examined phosphatidylserine stimulated histone H1 phosphorylation most efficiently. The effect of phosphatidylserine was not enhanced by 1,2-diolein. On the other hand, 1,2-diolein enhanced the phosphatidylinositol-dependent histone kinase activity, and lowered the K_a for Ca^{2+} in the presence of phosphatidylinositol. The enzyme activity was also stimulated by other phospholipids, including polyphosphoinositides, while phosphatidylcholine and sphingomyelin were without effect. Optimum enzyme activity was obtained at $100\ \mu\text{M}\ \text{CaCl}_2$ in the presence of phosphatidylserine and at $1\ \text{mM}$ in the presence of phosphatidylinositol. The Ca^{2+} - and phosphatidylserine-dependent histone H1 kinase reaction had a pH optimum at pH 6.5. Maximum enzyme activity was observed at $25\ \mu\text{M}$ ATP and at $100\ \mu\text{g}$ histone H1 per ml reaction mixture. The protamine kinase reaction had a pH optimum between 7.5 and 8.5. The optimum ATP concentration was $0.5\ \text{mM}$ and that of protamine $50\ \mu\text{g}$ per ml reaction mixture. In both reactions Mg^{2+} was the preferred divalent cation.

INTRODUCTION

Ca^{2+} is an important regulator of various intracellular activities [1,2,3], but the molecular mechanisms by which Ca^{2+} operates are known only in part. Recent evidence suggests that one mechanism is through the phosphorylation of specific proteins catalyzed by Ca^{2+} -dependent protein kinases [4]. Several such kinases which require calmodulin as a cofactor have been identified [5].

Another type of Ca^{2+} -dependent protein kinase that requires phospholipid as cofactor was identified by Takai et al. [6]. This enzyme is present in various organisms and tissues [7]. Due to the phospholipid-dependency of the kinase, membranes have been suggested to be a major site of action for this enzyme [6]. It has also been identified in membrane fractions [7-9].

We have shown earlier [10] that a major protamine-phosphorylating protein kinase in rat liver cytosol and microsomes is cyclic nucleotide independent. As is shown here this enzyme is Ca^{2+} - and phospholipid-dependent. The incomplete data concerning the properties of this enzyme available in the literature has prompted this characterization of the partly purified enzyme from rat liver cytosol. During the course of this work extensive purification of the Ca^{2+} - and phospholipid-dependent protein kinase from bovine heart and rat brain was reported [11,12], together with a characterization of the heart enzyme [13].

MATERIALS AND METHODS

Materials. Histone H1 (Type III-S),

protamine sulfate, 1,2-diolein, and 1,3-diolein were obtained from Sigma. [γ - ^{32}P] ATP was synthesized according to Chang et al. [14].

^{32}P -Inorganic phosphate was from the Radiochemical Centre, Amersham.

The phospholipids, dissolved in chloroform, were a generous gift from Dr. R. Sundler, Department of Physiological Chemistry, Lund. The chloroform was removed from the phospholipids by a stream of nitrogen. The phospholipids were suspended at 1 mg/ml in 10 mM Tris-HCl, pH 7.4, by sonication in a waterbath for 10 min. The synthetic peptide Leu-Arg-Arg-Ala-Ser-Val-Ala was a generous gift from Dr. Ö. Zetterqvist, Department of Medical Chemistry, Uppsala. All other chemicals were of reagent grade. The solutions were prepared in distilled water which had been filtered through a Milli-QTM system (Millipore).

Preparation of cytosol. Livers were obtained from male Sprague-Dawley rats (250-400 g), fasted over night. All operations were performed at 0-4°C. Livers were homogenized in 3 vol of buffer (50 mM Tris-HCl, pH 7.4, 0.35 M sucrose, 1 mM Na₂EDTA and 0.5 mM EGTA) with 6 strokes in a Potter-Elvehjem teflon-glass homogenizer. The homogenate was passed through 4 layers of surgical gauze and centrifuged at 10 000 xg for 10 min. The supernatant was then centrifuged at 95 000 xg for 90 min to obtain the soluble fraction. This was dialyzed for 2 h against 10 mM Tris-HCl and 0.5 mM EGTA. The dialysate was treated with 1 mM cyclic AMP for 30 min to dissociate the regulatory and catalytic subunits of the cyclic AMP-dependent protein kinases before chromatography on DEAE-Sephacel.

DEAE-Sephacel chromatography. A 1.5x30 cm column was packed with DEAE-Sephacel (Pharmacia) and equilibrated with 10 mM Tris-HCl, pH 7.4, and 0.5 mM EGTA. After application of the sample the column was washed with the same buffer also containing 0.1 mM cyclic AMP, before applying a linear 0-0.4 M KCl gradient in 10 mM Tris-HCl, pH 7.4, and 0.1 mM cyclic AMP. The total volume was 500 ml. Flow rate was 25 ml/h and fractions of 5 ml were collected. The eluate was analyzed for protein

kinase activity with protamine as substrate and for Ca^{2+} - and phospholipid-dependent protein kinase activity.

Analyses. Protein kinase analyses with protamine as substrate were performed as described earlier [15]. The incubation mixture contained in a total volume of 0.1 ml : 5 μmol Tris-HCl, pH 7.4, 1 μmol MgCl_2 , 30 μmol NaCl, 0.1 μmol dithioerythritol, 50 nmol [γ - ^{32}P] ATP (specific activity 50-100 cpm/pmol), 100 μg protamine, and an appropriate amount of enzyme protein. The reaction was carried out for 3-10 min at 25°C and stopped with 10 μl 12 M HCl. The mixture was then transferred quantitatively to filterpaper discs (Whatman 3 MM, 2.4 cm diameter) which were immersed in 5% (w/v) trichloroacetic acid/0.25% sodium tungstate solution. The discs were washed and analysed in a scintillation counter [15].

Ca^{2+} - and phospholipid-dependent protein kinase was measured essentially as described [7]. The assay mixture contained in a final volume of 0.1 ml : 2.5 μmol HEPES, pH 6.5, 1 μmol MgCl_2 , 10 μg histone H1, 50 nmol CaCl_2 , 3 μg phosphatidylserine, 0.5 or 5 nmol [γ - ^{32}P]ATP (specific activity 500-1000 cpm/nmol), and an appropriate amount of enzyme protein. The incubation time was 3 min at 25°C . The reaction was stopped with 10 μl 12 M HCl, and the mixture was transferred to filterpaper discs which were treated as described above.

Protein was determined by the Lowry method [17] in the presence of 3% sodium dodecylsulfate with bovine serum albumin as standard.

RESULTS

CaPK activity in rat liver cytosol.

The presence of Ca^{2+} - and phosphatidylserine-stimulated histone H1 kinase activity in rat liver cytosol was examined at 5 μM and 50 μM ATP (Table I). The phosphorylation obtained with histone H1 did not exceed the endogenous phosphorylation, except when both Ca^{2+} and phosphatidylserine were added. Then the specific activity was approximately 4 and 40 pmol phosphate incorporated per min per mg protein at 5 and 50 μM

ATP, respectively. With a recovery of 65 mg of cytosolic protein per g liver, this yields 2600 pmol incorporated per g liver at the higher ATP concentration. This is similar to the incorporation reported earlier [7] for rat liver cytosol when analyzed at 5 μ M ATP.

The phosphorylation of protamine at 50 μ M ATP was also examined. In the absence of Ca^{2+} this reaction was approximately 8 times faster than the Ca^{2+} - and phospholipid-dependent phosphorylation of histone H1. Ca^{2+} inhibited protamine phosphorylation by approximately 30%.

DEAE-Sephacel chromatography.

The cytosol was treated with cyclic AMP and chromatographed on DEAE-Sephacel. Three protein kinase peaks using protamine as substrate were resolved (Fig. 1). The first peak, which is rather small when the cytosol is chromatographed without pretreatment with cyclic AMP, has been identified earlier as the catalytic subunit of the cyclic AMP-dependent protein kinases [10] ; while the third peak is undissociated type II protein kinase. The kinase activity of the second peak was cyclic AMP-independent, and its position in the chromatogram remained unshifted by pretreatment of the cytosol with cyclic AMP. Analysis of the Ca^{2+} - and phosphatidylserine-dependent histone H1 kinase activity in the column eluate revealed one enzyme peak coinciding with this second protamine kinase peak.

The peak fractions were combined and chromatographed on hydroxylapatite (not shown). A major Ca^{2+} - and phospholipid-dependent histone H1 kinase peak eluted at 0.3 M ammonium phosphate trailing most of the proteins, while another small enzyme peak eluted earlier. Virtually all protamine kinase activity eluting from the column overlapped with the Ca^{2+} - and phospholipid-dependent enzyme.

The degree of purification of the Ca^{2+} - and phospholipid-dependent enzyme by these two steps was approximately 300-fold. The enzyme eluted from the hydroxylapatite column was, however, rather labile. It could not be stabilized by 34% sucrose [11] or 50% glycerol [12] as reported for other CaPK preparations. The characterization therefore had to be

performed with enzyme from the DEAE-chromatography step, that was stable at 0° for approximately 2 weeks. This enzyme had been purified 30-40 times over the cytosol, it had around half the specific activity of the bovine heart enzyme used for characterization earlier [11,13], and was stimulated more than 20-fold by Ca^{2+} (as compared to 8-fold for the bovine enzyme). The hydroxylapatite chromatography indicated that the enzyme obtained from DEAE-Sephacel was not contaminated substantially by other kinases using histone H1 or protamine as substrates. The enzyme was not inhibited by protein kinase inhibitor or stimulated by Ca^{2+} -calmodulin, and it gave only one peak of protamine kinase activity at pI 5.5 on isoelectric focusing. Furthermore, it catalyzed the phosphorylation of the peptide Leu-Arg-Arg-Ala-Ser-Val-Ala (a specific substrate for cyclic AMP-dependent protein kinases) at a 20-fold slower rate than protamine.

Effect of Ca^{2+} and phosphatidylserine on enzyme activity.

The effect of Ca^{2+} and phosphatidylserine, alone or in combination, on the phosphorylation of histone H1, protamine and endogenous components catalyzed by the enzyme preparation was examined (Table II). The phosphorylation of endogenous components was comparatively low, and increased slightly after addition of phosphatidylserine, particularly when Ca^{2+} was added also. The phosphorylation of histone H1, obtained by subtracting endogenous from total phosphorylation, was very low in the absence of phosphatidylserine. Ca^{2+} stimulated the reaction approximately 20-fold in the presence of phosphatidylserine. Protamine phosphorylation catalyzed by the enzyme was 5 times higher than the Ca^{2+} - and phosphatidylserine-dependent phosphorylation of histone H1. However, this reaction was inhibited markedly by Ca^{2+} and phosphatidylserine, with Ca^{2+} as the most potent inhibitor. Corresponding reactions catalyzed by the catalytic subunit of cyclic AMP-dependent protein kinase were also examined (Table II).

pH optimum.

Optimum activity of the Ca^{2+} - and phospholipid-dependent phosphorylation of histone H1 was between pH 6 and 7, and of protamine phosphorylation between pH 7.5 and 8.5 (Fig. 2). Accordingly, CaPK activity was measured at pH 6.5 and protamine kinase activity at pH 7.7. The optimum activity for the purified bovine brain enzyme was reported to be between pH 6 and 7 [11], while the purified rat brain enzyme had an optimum between pH 7.5 and 8.0 [12] when analyzed for CaPK-activity.

ATP concentration.

The dependency of the reaction rate on the ATP concentration was examined using protamine and histone H1 as substrates (Fig. 3). Maximum Ca^{2+} - and phosphatidylserine-dependent histone H1 kinase activity occurred at 25 μM ATP, while 500 μM ATP was required for maximum protamine kinase activity. The optimum ATP concentration for histone H1 kinase was much higher than the 5 μM used in earlier studies on CaPK [18]. It agreed well with the results reported for the bovine brain enzyme [13], however, but we did not observe the partial inhibition at high ATP concentrations.

Histone H1 and protamine concentrations.

The enzyme was incubated with increasing amounts of histone H1 at 5, 50, or 500 μM ATP, and in the presence or absence of Ca^{2+} and phosphatidylserine (Fig. 4). Maximum enzyme activity was observed at approximately 50, 100 and 300 μg histone per ml reaction mixture respectively, for the three ATP concentrations. It is notable that the basal activity (without added Ca^{2+} or phosphatidylserine) was elevated at histone concentrations above 500 μg per ml. Maximum activity for the enzyme with protamine as substrate and at 5 and 50 μM ATP was reached at 13 and 50 μg protamine per ml of reaction mixture, respectively (Fig. 5).

Effect of Ca^{2+} and phospholipids.

It has been reported that CaPK in addition to Ca^{2+} and phosphatidylserine requires unsaturated diacylglycerol (e.g. diolein) to exhibit full activity [19]. Diolein was reported not only to stimulate the activity of CaPK, but also to increase its affinity for Ca^{2+} . We therefore examined the phosphorylation of histone H1 at different concentrations of Ca^{2+} in the presence of phosphatidylserine or phosphatidylinositol alone, or together with 1,2-diolein (Fig. 6). Maximum activity with phosphatidylserine present was obtained at $100 \mu\text{M}$ Ca^{2+} , and the K_a for Ca^{2+} was $30 \mu\text{M}$. Ca^{2+} concentrations above 1 mM were inhibitory. The simultaneous addition of 1,2-diolein did not affect the maximum kinase activity, and lowered the K_a only slightly. At low Ca^{2+} concentrations (up to $10 \mu\text{M}$), however, 1,2-diolein was markedly stimulatory. Maximum kinase activity with phosphatidylinositol was observed at 2 mM CaCl_2 . 1,2-Diolein added in combination with phosphatidylinositol increased the enzyme activity 3 times up to the level obtained with phosphatidylserine, and decreased the K_a for Ca^{2+} from 0.6 mM to 0.2 mM . 1,2-Diolein alone had no effect on kinase activity at any concentration of Ca^{2+} .

The effect of several different phospholipids on the histone H1 kinase activity was examined in the presence or absence of 1,2-diolein at $500 \mu\text{M}$ CaCl_2 (Table III). The enzyme was most active in the presence of phosphatidylserine, followed by phosphatidylinositol, polyphosphoinositides, phosphatidic acid and phosphatidylethanolamine. The enzyme was inactive with phosphatidylcholine and sphingomyelin and showed a low activity with lysophosphatidylethanolamine, particularly in the absence of 1,2-diolein. 1,2-Diolein stimulated enzyme activity most profoundly in combination with phosphatidylinositol, and to a smaller extent with phosphatidylethanolamine and phosphatidic acid.

DISCUSSION

The degree of purification of rat liver CaPK obtained by the DEAE-Sephacel step (30-40-fold) was approximately 5 times higher than that reported for the bovine heart enzyme [11]. This degree of purification is unusually high for DEAE-Sephacel chromatography. We also noted that the recovery of CaPK exceeded 200%. These observations suggested that the cytosolic CaPK was in a partly inhibited state prior to chromatography, and we have shown in preliminary experiments that a heat-labile factor in the cytosol will inhibit purified CaPK. The nature of this factor, and the question whether it is analogous to the inhibitor of cyclic AMP-dependent protein kinases has not been examined yet.

The partially purified CaPK from rat liver cytosol exhibited properties similar to those reported for the bovine heart enzyme [11,13], whereas a comparison with rat brain CaPK [12] has to await its characterization.

Rat liver CaPK examined here has been isolated and partly characterized earlier as a protamine-phosphorylating enzyme [10]. This enzyme was present both in the cytosol and in microsomes, and the enzyme isolated from these sources had similar properties.

When protamine was used as substrate the purified CaPK was inhibited both by Ca^{2+} and phosphatidylserine, and the inhibition by these agents was additive. In contrast, the phosphorylation of histone H1 was dependent on both Ca^{2+} and phosphatidylserine. If the phospholipid was omitted there was no enzyme activity, while the activity was low without Ca^{2+} but in presence of phospholipid. The enzyme-catalyzed phosphorylation of protamine and histone H1 also differed in their dependency on the concentration of ATP. A 20-fold higher concentration was required for a maximum rate of protamine phosphorylation than of histone phosphorylation. A third difference between these two reactions was in their pH dependency.

It is unclear so far whether these differences in the phosphorylation of protamine and histone H1 reflect different enzymic mechanisms, or whether they primarily are due to properties imposed by the two substrates. It is not unlikely, for instance, that the extremely basic protamine will perturb phospholipid-enzyme interactions, resulting in a reaction independent of or even slightly inhibited by Ca^{2+} and phospholipid. Probably there is an interaction between a Ca^{2+} - and phospholipid-binding domain in CaPK and the catalytic site, and if this interaction is disturbed by protamine the properties of the phosphorylation reaction will change. In the original work by Takai et al. [20] CaPK was described as an inactive proenzyme which could be activated by proteolysis to yield a Ca^{2+} - and phospholipid-independent enzyme. Presumably the catalytic properties of this latter enzyme are similar to those imposed by protamine. The alternative, that histone H1 and protamine are phosphorylated by different catalytic sites of the enzyme appears less likely.

The function of CaPK in liver cells is still enigmatic. Only around 30% of the enzyme is soluble, while 70% is attached to membranes. More than 90% of the particulate enzyme is found in microsomes, whereas the remainder is associated with plasma membranes [9]. The distribution of CaPK between soluble and particulate fractions appears to be tightly regulated, at least in parietal yolk sac cells where the enzyme is translocated from the soluble fraction to plasma membranes if cells are treated with phorbol esters [21]. In addition, we have observed a substantial loss (up to 50%) in liver microsomal CaPK upon treatment of rats with phenobarbital (unpublished). Apart from these observations, nothing is known about external factors influencing the subcellular distribution of CaPK.

Ca^{2+} is an intracellular factor that may influence CaPK in two ways, either as an activator of the soluble enzyme together with diolein and/or phospholipid, or as a mediator of enzyme binding to membranes. The latter function of Ca^{2+} was originally suggested by Takai et al [6], and was supported by our observation that Ca^{2+} is required for the binding of CaPK to Ca^{2+} -depleted microsomal membranes in vitro [22].

The possible physiological importance of diolein (or rather of diacylglycerol that is formed in the cell by phospholipase C hydrolysis of phospholipids) is still unresolved. It was suggested that diolein together with Ca^{2+} would facilitate the binding of CaPK to membranes [19] , but our in vitro binding studies 22 do not support this suggestion. Diolein might also play a role in the activation of cytosolic CaPK since it lowers the concentration of Ca^{2+} required in the phospholipid-dependent activation of the enzyme [19] . It should be pointed out, however, that the concentration of free phospholipids required for such an activation probably is much higher than the physiological concentration in the cytosol. Nevertheless, protein substrates for CaPK are likely to be present in the cytosol, since endogenous soluble proteins of rat liver and other tissues can be phosphorylated in a Ca^{2+} - and phospholipid-dependent reaction [18] . None of these liver proteins have been identified to date, and therefore the physiological importance of rat liver soluble CaPK cannot be assessed.

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TABLE I. PROTEIN KINASE ACTIVITY IN RAT LIVER CYTOSOL.

Protein kinase activity was analyzed with histone H1 or protamine as substrates in the presence or absence of CaCl_2 and phosphatidylserine (PS) as indicated. The endogenous incorporation of phosphate into cytosolic components was also measured. The figures are averages of three measurements $\pm$ S.E.

Substrate	Protein kinase activity (pmol/min per mg protein)		
	-	+	+ CaCl_2 + PS
5 μM ATP			
Histone H1	6.7 $\pm$ 1.3	6.3 $\pm$ 1.4	12.7 $\pm$ 1.2
Endogenous	6.0 $\pm$ 1.8	6.0 $\pm$ 1.2	8.8 $\pm$ 1.3
50 μM ATP			
Histone H1	22.8 $\pm$ 2.3	20.7 $\pm$ 2.7	68.1 $\pm$ 10.9
Endogenous	23.3 $\pm$ 2.8	24.3 $\pm$ 3.9	27.0 $\pm$ 2.9
Protamine	322 $\pm$ 60	217 $\pm$ 46	223 $\pm$ 35
		303 $\pm$ 53	

TABLE II. THE EFFECT OF Ca^{2+} AND PHOSPHATIDYL SERINE ACTIVITY

The Ca^{2+} - and phospholipid-dependent protein kinase and the catalytic subunit of cyclic AMP-dependent protein kinase were purified through the DEAE-Sephacel stage. Protein kinase activity was measured with the indicated substrates.

Substrate	Ca^{2+} - and phospholipid-dependent protein kinase (pmol/min per mg protein)				Cyclic AMP-dependent protein kinase (pmol/min per mg protein)	
	-	+	-	+	-	CaCl_2 PS
Histone phosphorylation						
Total	75	65	148	1414	105	79
Endogenous	65	56	88	109	20	14
Histone H1	10	9	60	1305	85	65
Protamine phosphorylation						
Total	6515	4349	5917	3628	432	391
Endogenous	62	46	68	82	4	4
Protamine	6453	4313	5845	3546	428	387

TABLE III. EFFECT OF PHOSPHOLIPIDS AND DIOLEIN ON CaPK ACTIVITY

The enzyme was incubated with 3 μ g of the indicated lipids in the presence or absence of 1.5 μ g 1,2 diolein. The activities were measured at 0.5 mM CaCl_2 as the increase in histone H1 phosphorylation on addition of the lipid.

Lipid	Protein kinase activity (pmol/min per mg protein)	
	-	+ 1,2 diolein
Phosphatidylserine	724	671
Phosphatidylinositol	144	456
Phosphatidylinositol monophosphate	375	423
Phosphatidylinositol diphosphate	211	294
Phosphatidyl- ethanolamine	75	138
Phosphatidylcholine	0	0
Sphingomyelin	0	6
Phosphatidic acid	153	221
Lysophosphatidyl- ethanolamine	2	55
1,3 Diolein	12	7
1,2-Diolein	4	1

LEGENDS TO FIGURES

- Fig. 1. Fractionation of protein kinases from rat liver cytosol by DEAE-Sephacel chromatography. Conditions used were as described in the Methods section. Protein kinase activity was measured with protamine as substrate (●), or with histone H1 as substrate (▲). In the latter case the values were corrected for the activity seen in the absence of 500 μM CaCl_2 . ----, Protein concentration measured according to Lowry; _____, KCl gradient. The applied amount of protein was 430 mg. The kinase activities are expressed as nmol phosphate incorporated per min.
- Fig. 2. The effect of pH on protein kinase activity. The Ca^{2+} - and phosphatidylserine-dependent phosphorylation of histone H1 (●) and protamine kinase (▲) were measured as described in Methods. The activities are expressed as nmol phosphate incorporated per mg protein and min. The buffers used were sodium acetate (pH 5.6-6.4), HEPES (pH 6.4-8.4), and sodium carbonate (pH 8.4-9.6).
- Fig. 3. The effect of ATP on protein kinase activity. Protein kinase activity was measured with protamine (▲) or histone H1 (●) as substrate and expressed as nmol phosphate incorporated per mg protein and min. In the latter case the values were corrected for the activity seen in the absence of 500 μM CaCl_2 .
- Fig. 4. The effect of histone H1 concentration on CaPK activity. The CaPK activity was measured at different histone H1 concentrations using the 5 (◻), 50 (Δ) or 500 (o) μM , and expressed as nmol phosphate incorporated per mg protein and min. Closed symbols is phosphorylation in the presence of CaCl_2 and phosphatidylserine, open symbols is in the absence of these compounds.

Fig. 5. The effect of protamine on protein kinase activity. Protein kinase analyses were performed with protamine as substrate with 5 (o) or 50 (●) μ M ATP. The activities are expressed as nmol phosphate incorporated per mg protein and min.

Fig. 6. Effects of Ca^{2+} , phosphatidylserine, phosphatidylinositol and 1,2-diolein on CaPK activity. Histone H1 phosphorylation was measured at different CaCl_2 concentrations in the presence of phosphatidylserine (o) or phosphatidylinositol (Δ). Closed symbols indicate that 1,2-diolein was also included in the reaction medium. The Ca^{2+} concentrations indicated represented added CaCl_2 , not necessarily the actual, free Ca^{2+} present in the incubation medium. The activities are expressed as nmol phosphate incorporated per mg protein and min.

FIG. 1

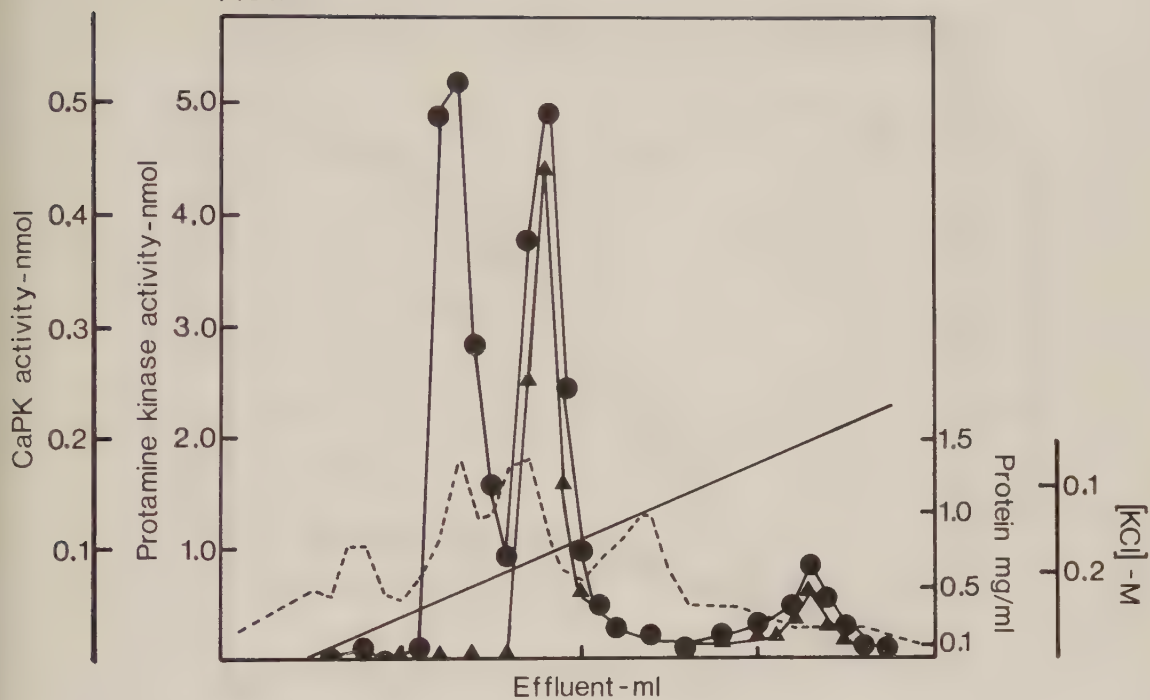


FIG. 2

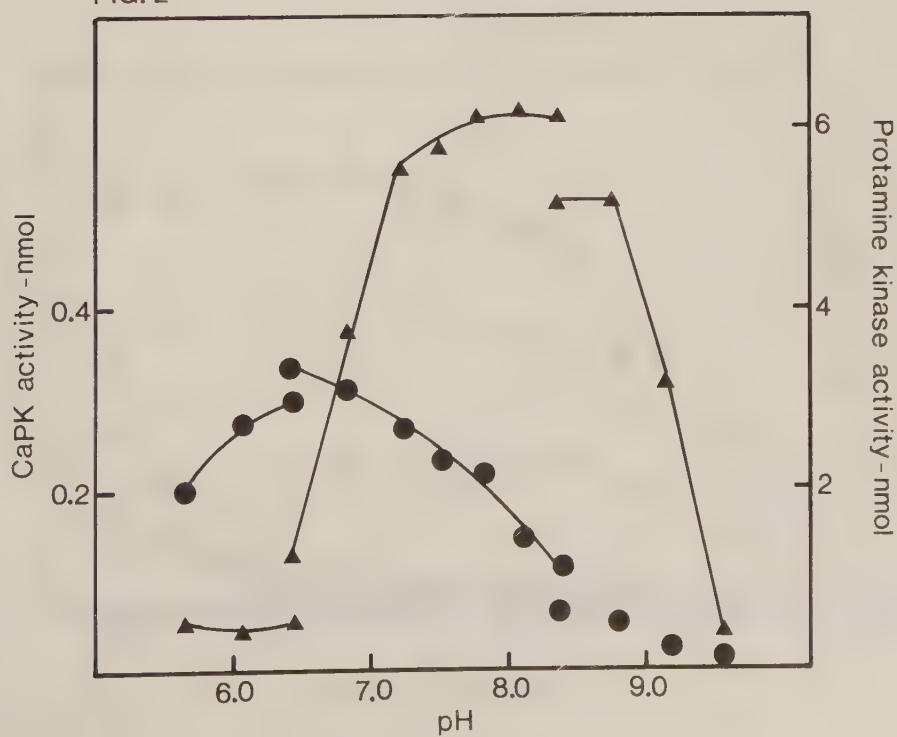


FIG 3

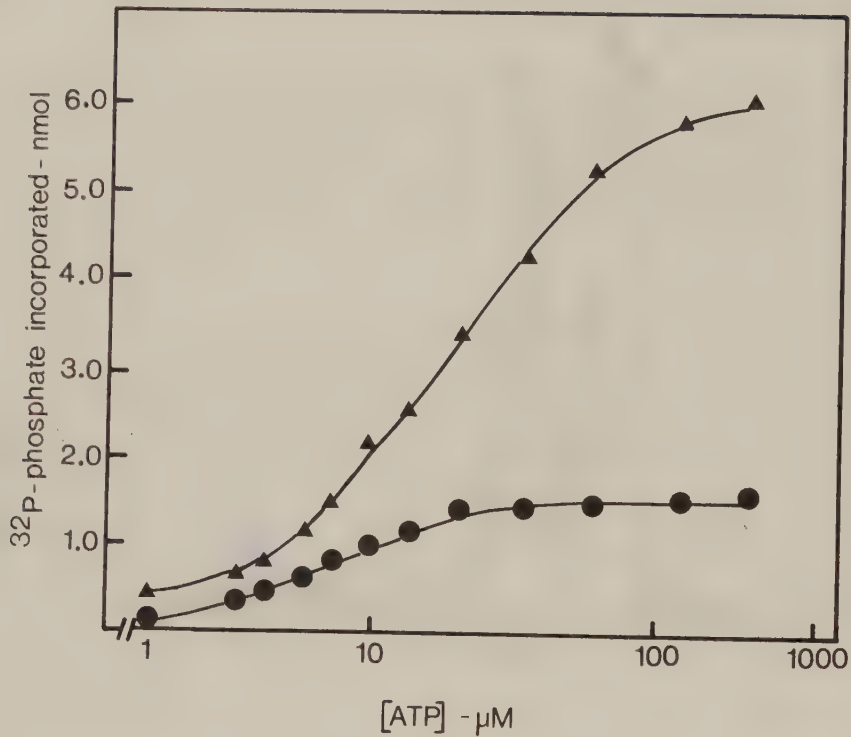


FIG 4

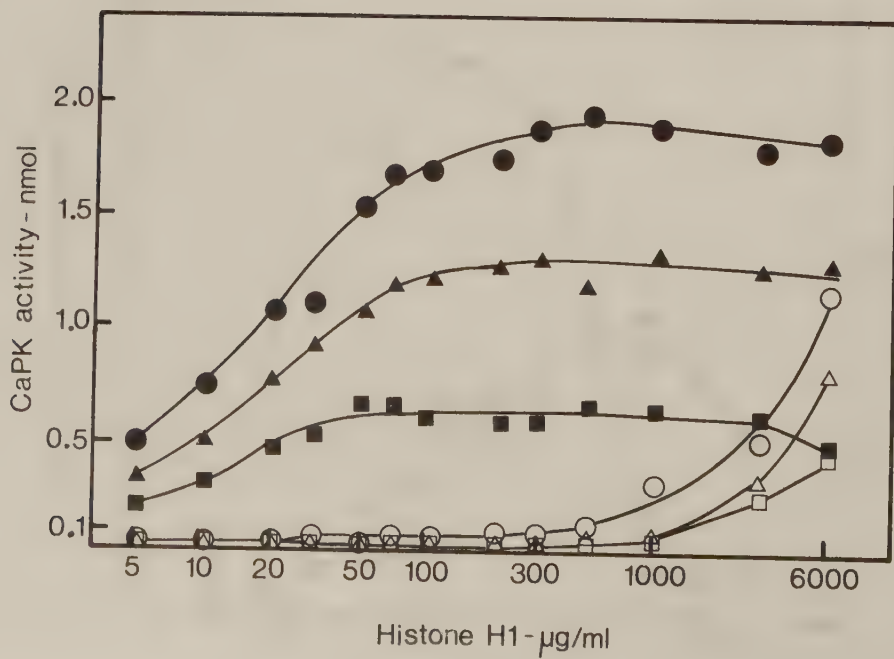


FIG.5

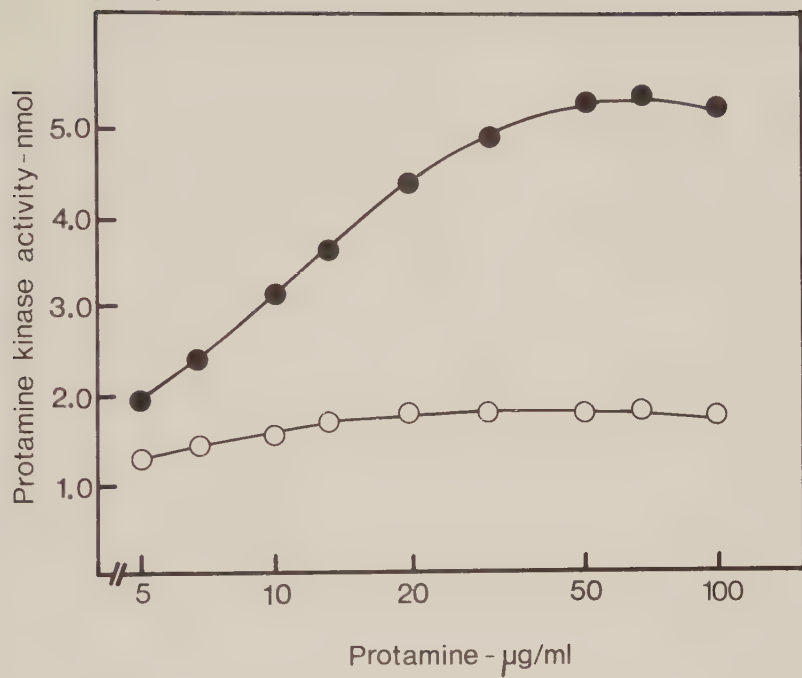
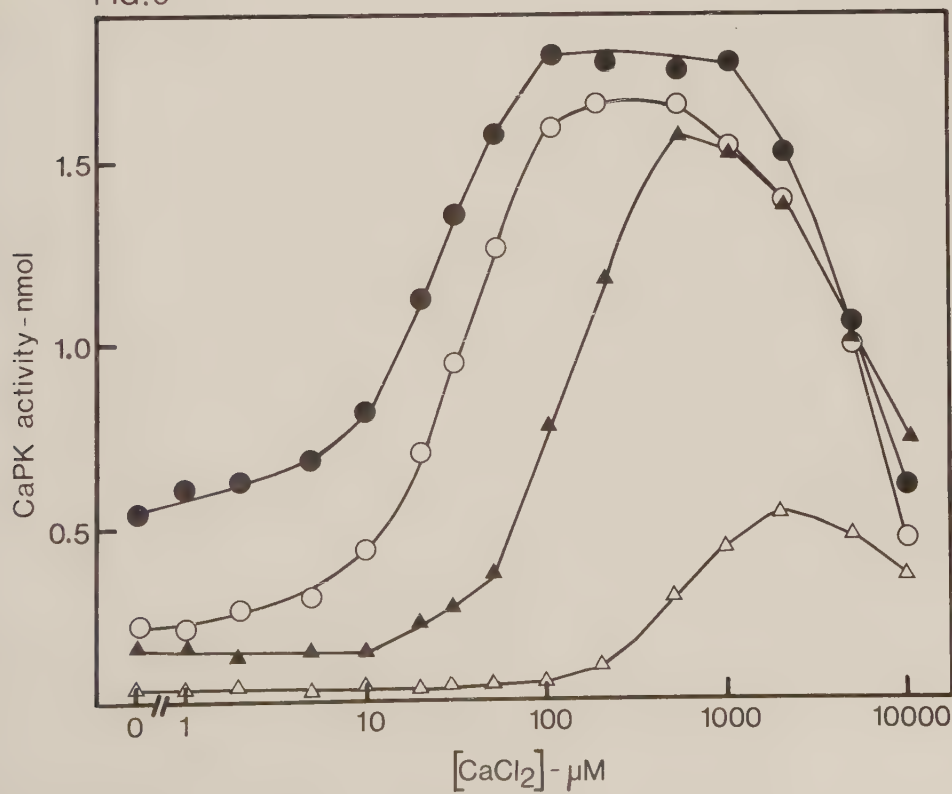


FIG.6



DETERMINATION OF Ca^{2+} - AND PHOSPHOLIPID-DEPENDENT PROTEIN KINASE
IN RAT LIVER MEMBRANES

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Abbreviations used: CaPK, Ca^{2+} - and phospholipid-dependent protein kinase; octylglycoside, n-octyl- β -D-glucopyranoside.

SUMMARY

A method has been developed to measure the Ca^{2+} - and phospholipid-dependent protein kinase in membrane fractions. The method is based on the fact that this enzyme is resistant to comparatively high concentrations of octylglycoside. Rat liver membranes were treated with octylglycoside and the phosphate incorporation from [γ - ^{32}P] ATP was measured in the presence of histone H1. The enzyme activity was determined as the difference between the incorporation obtained after addition of Ca^{2+} and phosphatidylserine and the incorporation obtained without these additions but with EGTA. The endogenous incorporation of phosphate to membrane components was constant under these incubation conditions. The conditions for determination of the membrane-bound enzyme were optimized. Two thirds of the total enzymic activity was attached to membranes in rat liver cells. A highly purified plasma membrane preparation had the highest specific activity, while most of the bound enzyme was found in microsomes, and only traces were found in mitochondria.

INTRODUCTION

The Ca^{2+} - and phospholipid-dependent protein kinase (CaPK), originally described by Takai et al. [1] is widely distributed in various organisms and tissues [2,3]. The phospholipid dependency of the enzyme has been taken to indicate that it may bind to cellular membranes [1] under appropriate conditions. Support for this suggestion has come from the recent finding that CaPK apart from being present in the cytosol also is attached to membrane fractions in various tissues [2,4].

One difficulty which has hampered studies on membrane-bound CaPK, for instance studies designed to examine possible regulatory mechanisms for the binding of CaPK to membranes, is the lack of a convenient assay procedure for the bound enzyme. The membrane-bound enzyme has been measured in the high-speed supernatant after extraction with EGTA and Triton X-100 [4]. Apart from the time-consuming centrifugation step, this procedure is limited by the inhibiting effect of Triton X-100 on CaPK activity. Detergent concentrations above 0.0075% are inhibitory [4], and this is far below what is required to solubilize the enzyme, necessitating extensive dilution of the extracts before enzyme analysis.

In this paper we describe a simple and sensitive procedure for analysis of CaPK bound to membranes. The procedure is based on the fact that the enzyme tolerates the detergent octylglycoside rather well. The conditions for analysis have been optimized, and the kinase activity in rat liver membrane fractions determined.

EXPERIMENTAL

Materials. n-Octyl- β -D-glucopyranoside (octylglycoside); histone H1 and protamine sulfate were obtained from Sigma Chemicals Co. [γ - ^{32}P] ATP was synthesized according to Chang et al. [5]. Phosphatidylserine was a generous gift from Dr. R. Sundler, Department of Physiological Chemistry, Lund. All solutions were made up in double distilled water filtered through a Milli-QTM system (Millipore).

Preparation of liver membrane fractions. These were prepared from male Sprague-Dawley rats which had been starved for 18 h before sacrifice. Microsomes were prepared either in the absence or presence of 1 mM EDTA and 0.5 mM EGTA. Livers were homogenized in 3 vol per g tissue of 50 mM Tris-HCl, pH 7.4, and 0.35 M sucrose, with or without the chelators, by six strokes in a glass-teflon Potter-Elvehjem homogenizer. The homogenate was centrifuged at 9 000 xg for 10 min, and the resulting supernatant at 105 000 xg for 90 min. The microsomal pellet was washed once in buffer alone. Smooth microsomes were obtained from the microsomal pellet by suspension in the homogenization medium and discontinuous sucrose gradient centrifugation [6] with or without 1 mM EDTA and 0.5 mM EGTA. The 0.9 M/1.3 M sucrose interface was aspirated, diluted with 10 mM Tris-HCl, pH 7.4, and made 0.3 M in KCl before centrifugation for 90 min at 105 000 xg. The resulting pellet was washed once in 10 mM Tris-HCl, pH 7.4, and resuspended in the same buffer.

Plasma membrane-enriched fractions were prepared according to Aronson and Touster [7]. Two fractions were obtained; the N_2 -fraction primarily contains bile canalicular membranes [8], while the P_2 -fraction is a mixture of blood sinusoidal membranes and Golgi [8]. Mitochondria were prepared according to Greenawalt [9]. The membrane fractions were suspended in 10 mM Tris-HCl, pH 7.4. Rat liver cytosol was obtained as the 105 000 xg supernatant of homogenates prepared in the presence of 1 mM EDTA and 0.5 mM EGTA. It was chromatographed through Sephadex G-25 before use.

Protein was determined by the Lowry [10] procedure in the presence of 3% (w/v) sodium dodecylsulfate with bovine serum albumin as standard.

Protein kinase analyses. The Ca^{2+} - and phospholipid-dependent protein kinase was analyzed in a mixture containing (unless otherwise is indicated) 2.5 μ mol HEPES, pH 6.5, 1 μ mol $MgCl_2$, 5 nmol [γ - ^{32}P] ATP (specific activity 400-800 CPM/pmol), 10 μ g histone H1, 15-30 μ g membrane protein, 0.175 mg octylglycoside and either 10 nmol EGTA, or 25 nmol $CaCl_2$ and (optional for membrane fractions) 5 μ g phosphatidylserine; the volume was 100 μ l. Membranes were preincubated with detergent for at least 5 min before starting the reaction with ATP. Incubations were for 3 min

at 25°C, and were stopped by the addition of 10 μ l 12 M HCl. The reaction mixtures were quantitatively transferred to filter paper discs, which were washed and analyzed in a scintillation counter as described earlier [11]. Endogenous phosphorylation was measured in the same way but omitting histone H1. The kinase activity was obtained as the Ca^{2+} - and phospholipid-dependent increase in phosphorylation of histone H1 after subtraction of endogenous phosphorylation.

Soluble CaPK from rat liver cytosol was partially purified by chromatography on DEAE-Sephacel (Sommarin, M. and Jergil, B., unpublished). The preparation was virtually free from cyclic AMP-dependent protein kinase activity. Protamine kinase activity was measured as described [11] omitting NaCl. All analysis were performed in duplicate.

RESULTS

The effect of detergents on CaPK activity

Soluble CaPK is characterized by its dependency on Ca^{2+} and phospholipids for full activity when histone H1 is used as substrate [1]. The enzyme also phosphorylates protamine efficiently in a Ca^{2+} - and phospholipid-independent manner [12]. The effect of detergent on CaPK and protamine kinase activities was studied using partially purified enzyme from rat liver cytosol (Fig. 1). Triton X-100 at concentrations below 0.01% slightly stimulated CaPK activity. This stimulation ranged from 25% to 90% in different experiments. Above 0.01% Triton was strongly inhibitory, thus corroborating earlier results [4]. In a search for a less inhibitory detergent, we found that octylglycoside was the only one examined which could be used with advantage. Indeed, CaPK entirely retained its Ca^{2+} - and phospholipid-dependent activity in the presence of 0.25% octylglycoside (Fig. 1), i.e. at a 25-fold higher concentration than with Triton X-100. When the concentration was increased further the activity decreased rapidly. At concentrations below 0.25% the enzyme was slightly stimulated (up to 30%). The enzyme also retained its activity after treatment with high concentrations of octylglycoside, provided that the extract was properly diluted before analysis (cf below).

The influence of Triton X-100 and octylglycoside on the Ca^{2+} - and phospholipid-independent phosphorylation of protamine by the enzyme was also examined (Fig. 1). In this case the enzyme was still fully active in the presence of more than 1% (Triton X-100) or close to 1% (octylglycoside) of the detergents.

Octylglycoside concentrations

The effect of octylglycoside on membrane-bound protein kinase was examined next. To this end smooth microsomes were preincubated with various concentrations of octylglycoside and then diluted tenfold in the CaPK reaction mixture. No enzyme activity could be detected in the absence of detergent. After addition of octylglycoside a Ca^{2+} - and phospholipid-dependent histone H1 kinase activity was observed, however. The activity increased with increasing concentrations of detergent (Fig. 2) reaching a maximum in the concentration range from 0.12%, i.e. the membranes had been pretreated with 1.2 to 2.3% (w/v) of octylglycoside. The lower activity observed at 0.3% octylglycoside was due to inhibition of the enzyme at this concentration and could be overcome through a further dilution before analysis (not shown). If smooth microsomes were mixed directly with a reaction mixture containing 0.175% octylglycoside virtually no Ca^{2+} - and phospholipid-dependent kinase activity could be detected. This shows that solubilization of the membrane, at least in part, is a prerequisite for measuring bound activity.

Effect of Ca^{2+} and phospholipids

The Ca^{2+} dependency of histone H1 phosphorylation by smooth microsomes treated with octylglycoside was examined. A maximum rate of phosphorylation was obtained at approximately 100 μM Ca^{2+} (Fig. 3). Without added Ca^{2+} the rate was still comparatively high and around half of that at the optimum concentration. This could be diminished further, however, by the addition of 50 μM EGTA, yielding a basal rate of histone phosphorylation that was approximately 20% of the rate at optimum conditions. This basal rate corresponds well to the histone H1 phosphorylation expected from the cyclic AMP-dependent protein kinase present in smooth microsomes [13]. The partial activation of the enzyme in the absence of EGTA was probably due to the release of Ca^{2+} from microsomal vesicles on solubilization

with octylglycoside; microsomes together with mitochondria contain the major intracellular pool of Ca^{2+} .

Further aspects on the effects of Ca^{2+} and EGTA, together with the effect of phosphatidylserine, on the incorporation of phosphate into endogenous membrane components and histone H1 are shown in Table I. None of these additions had any appreciable effect on the phosphorylation of endogenous components. Furthermore, the addition of phosphatidylserine did not increase the incorporation of phosphate into histone H1. Thus, there is a sufficiently high endogenous concentration of phospholipids in the membrane extract to obtain optimum CaPK activity provided that the observed enzyme is phospholipid-dependent. The enzyme activity most likely represents CaPK, since the only protein kinase fraction obtained by chromatography of microsomal extracts on DEAE-cellulose that phosphorylates histone H1 in a Ca^{2+} -dependent manner also is phospholipid-dependent (Jergil, B., unpublished).

ATP concentration

Usually 5 μM ATP has been used for the analysis of CaPK [1,2]. This is far from a saturating concentration for the microsomal enzyme which phosphorylates histone H1 most actively at 50 μM ATP (Fig. 4). At this concentration the activity is 3.5 times higher than with 5 μM ATP.

Histone H1 concentration

The effect of variations in the concentration of histone H1 on phosphate incorporation is presented in Fig. 5. Maximum incorporation was obtained at 100 μg per ml incubation medium when Ca^{2+} and phosphatidylserine were present. Without addition of these compounds the incorporation increased up to 30 μg histone per ml and was then constant.

Other parameters

We have also tested several other parameters using smooth microsomes. Mg^{2+} is necessary for the phosphorylation of histone H1 and maximum activity is obtained at 5 mM concentration. The pH optimum for the reaction is around pH 6.5. These properties together with the saturation curves for

ATP and histone H1 concentrations are consistent with those found for partially purified CaPK from rat liver cytosol (Sommarin, M. and Jergil, B., unpublished observations).

The phosphorylation is proportional to the amount of membrane up to 30 μ g of membrane protein per incubation for smooth and unfractionated microsomes, and up to 10 μ g for plasma membranes. Using these amounts of membranes the reaction is linear with time for 10 and 3 min for smooth microsomes and plasma membranes, respectively. We have also added various amounts of cytosolic CaPK to smooth microsomes before analysis under standard conditions and found the expected activities within a wide range of enzyme concentrations.

CaPK activities in subcellular fractions

The Ca^{2+} - and phosphatidylserine-dependent phosphorylation of histone H1 catalyzed by various rat liver subcellular fractions is shown in Table II. The highly purified plasma membrane N_2 -fraction had the highest specific kinase activity, approximately twice that of the less homogeneous P_2 -fraction. Both smooth and unfractionated microsomes showed a slightly lower activity when prepared in the absence rather than in the presence of EDTA and EGTA. The specific activity of these fractions was 6-10 times higher than that found in the cytosol. The activity of mitochondria was close to the detection limit.

DISCUSSION

The results presented show that the Ca^{2+} - and phospholipid-dependent phosphorylation of histone H1 catalyzed by membrane-bound protein kinase can be measured directly after treatment of membranes with octylglycoside. The enzyme is much less sensitive to this detergent than to other detergents, including Triton X-100 which has been used earlier to extract CaPK from membranes [4]. As a result no extensive dilution of the detergent-treated material had to be done before enzyme analysis. Centrifugation to remove particulate material after the addition of detergent, thereby diminishing the substantial endogenous incorporation of phosphate, and hence the Ca^{2+} -independent phosphorylation, could also be avoided. The

endogenous phosphorylation was 2-4 times lower (depending on the membrane material examined) in the high-speed supernatant than in the membrane-detergent mixture before centrifugation. There was no difference in the Ca^{2+} - and phospholipid-dependent phosphorylation of histone H1 catalyzed by the supernatant and the mixture, unless the solubilization of the enzyme was incomplete. In fact, more than 95% of the enzyme was solubilized from microsomes by 1.2% octylglycoside, the detergent concentration yielding the highest kinase activity in membrane-detergent mixtures.

The incorporation of phosphate into histone H1 is catalyzed by at least two enzymes present in membrane fractions. The catalytic subunit of cyclic AMP-dependent protein kinase, presumably present on the luminal surface of microsomes [13] and the external surface of plasma membranes [14], will phosphorylate histone H1 in a Ca^{2+} - and phospholipid-independent manner. The phosphorylation observed without addition of these substances is approximately that expected from the amount of catalytic subunit present in the membrane fractions. The Ca^{2+} -dependent phosphorylation of histone H1 is most likely catalyzed by CaPK, although the phospholipid-dependency is masked by endogenous phospholipids. It seems less likely that this reaction should be due to other Ca^{2+} -dependent protein kinases, since a Ca^{2+} - and phospholipid-dependent enzyme (corresponding to peak MII in ref. 13) can be recovered after chromatography of microsomal extracts on DEAE-cellulose. The membrane-bound enzyme also has the same catalytic properties as partially purified CaPK from rat liver cytosol (Sommarin, M. and Jergil, B., unpublished data).

The endogenous components phosphorylated in membranes include protein [15,16] as well as polyphosphoinositides that are only partly removed in the analytical procedure used. Since the endogenous incorporation of phosphate was constant in incubations with and without Ca^{2+} and phosphatidylserine (this applies to all membrane preparations examined), the endogenous phosphorylation can be omitted from the calculations of CaPK activity. Thus, the enzyme activity is the difference between the total incorporation of phosphate obtained with added Ca^{2+} and (optional) phos-

phatidylserine and that obtained without these additions but with EGTA. The presence of 100 μ M EGTA together with Ca^{2+} does not affect the Ca^{2+} -dependent phosphorylation under standard conditions. The endogenous phosphorylation has been included in all enzyme determinations presented in this paper, however, in order to check its extensiveness under various incubation conditions.

One problem concerning the accuracy of the determination of membrane-bound CaPK is the rather high activity obtained without addition of Ca^{2+} and phosphatidylserine. This amounts to approximately 25% in microsomal fractions prepared in the presence of chelators and the N_2 -fraction, and to around 50% in microsomes prepared without chelators and the P_2 -fraction. We have obtained good correlation, however, between repetitive analyses of various membrane preparations. The Ca^{2+} -dependent increases in membrane kinase activities found here were also substantially larger than those observed earlier [4] in corresponding guinea pig heart membranes after extraction with Triton X-100. Although we obtained similar Ca^{2+} -dependent protein kinase activities in microsomes after treatment with octylglycoside and Triton X-100, the Ca^{2+} -independent incorporation of phosphate was more than two times higher with Triton X-100.

As to the distribution of CaPK in liver cells approximately two thirds of the total activity is found in the microsomal fraction and around one third in the cytosol, assuming a recovery of 15 and 65 mg of protein per g tissue in the two fractions, respectively. Only a few per cent of the activity resides in plasma membranes, although this fraction shows the highest specific activity of the enzyme. The total incorporation of phosphate catalyzed by the enzyme is approximately 7.5 nmole incorporated/min per g liver tissue. This is nearly 4 times more than was reported previously [2], but corresponds rather well with the very recent results by Kikkawa et al. [17]. These latter authors found, however, that only 20% of the liver CaPK resided in the particulate fraction.

One question regards the possibility that CaPK becomes bound to membranes after homogenization of the tissue. We have found in binding experiments of partially purified cytosolic CaPK and microsomes that

the enzyme will only bind to the membranes if Ca^{2+} is also added. This, together with the finding that the activity in smooth and unfractionated microsomes is slightly higher after homogenization in the presence rather than absence of chelators, suggests that the enzyme largely exists in a bound state in the intact liver cell.

However, so far it is not known whether any specific factor(s) determines the subcellular distribution of CaPK. It should be possible to examine this question further using the described procedure for determination of CaPK bound to membranes.

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TABLE I

THE EFFECT OF Ca^{2+} , EGTA AND PHOSPHATIDYLSERINE ON THE PHOSPHORYLATION OF HISTONE H1 BY SMOOTH MICROSOMES

Smooth microsomes were prepared in the presence of EDTA and EGTA. The incorporation of ^{32}P -phosphate was measured with (total) or without (endogenous) addition of histone H1. The difference is taken as a measure for the incorporation into histone H1. Incubations were performed as described in Methods with additions as indicated (250 μM CaCl_2 , 5 μg phosphatidylserine (PS), 100 μM EGTA).

Substrate	^{32}P -phosphate incorporation (nmol/min per mg protein)					
	-	-	-	-	+	+ CaCl_2
	-	-	+	+	-	+ PS
	-	+	-	+	-	- EGTA
Total	0.142	0.088	0.164	0.085	0.266	0.271
Endogenous	0.057	0.053	0.052	0.052	0.042	0.045
Histone H1	0.085	0.035	0.112	0.033	0.224	0.226

TABLE II

Ca²⁺- AND PHOSPHOLIPID-DEPENDENT ACTIVITIES IN RAT LIVER SUBCELLULAR FRACTIONS.

The microsomal fractions were prepared in the absence (-) or presence (+) of EDTA and EGTA. The data represent means $\pm$ S.E. for the number of determinations given.

Fraction	Specific activity (nmol/min x mg protein)	n
Smooth microsomes		
-EDTA/EGTA	0.239 $\pm$ 0.059	5
+EDTA/EGTA	0.397 $\pm$ 0.077	6
Unfractionated microsomes		
-EDTA/EGTA	0.242 $\pm$ 0.113	6
+EDTA/EGTA	0.290 $\pm$ 0.048	6
Plasma membranes		
N ₂ -fraction	1.25 $\pm$ 0.20	4
P ₂ -fraction	0.542 $\pm$ 0.100	4
Mitochondria	<0.015	2
Cytosol	0.041 $\pm$ 0.008	3

LEGENDS TO FIGURES

Fig. 1. The effect of detergents on protein kinase activity. Partially purified CaPK from rat liver cytosol (12 μg per sample) was analyzed for Ca^{2+} - and phospholipid-dependent phosphorylation of histone H1 (closed symbols) or protamine phosphorylation (open symbols) in the presence of various concentrations of Triton X-100 (Δ) or octylglycoside (o). The phosphate incorporation is expressed as pmol per min.

Fig. 2. Ca^{2+} - and phospholipid-dependent phosphorylation of histone H1 by smooth microsomes at various concentrations of octylglycoside. Smooth microsomes (30 μg of membrane protein) were preincubated with octylglycoside for 5 min before a 10-fold dilution in the kinase reaction mixture to the indicated concentrations. The open circle shows the kinase activity when measured directly at 0.175% octylglycoside without preincubation at a high detergent concentration.

Fig. 3. The effect of Ca^{2+} on histone H1 phosphorylation by smooth microsomes. Kinase activity was measured as described in Methods with 40 μg of membrane protein at different Ca^{2+} concentrations, or after the addition of 50 μM EGTA. The incorporation of phosphate is expressed as pmol per min.

Fig. 4. Histone H1 phosphorylation by smooth microsomes at different ATP concentrations. Kinase activity was measured as described in Methods using 30 μg of membrane protein.

Fig. 5. Histone H1 phosphorylation by smooth microsomes at different histone concentrations. The histone H1-dependent phosphorylation was measured using 40 μg of membrane protein in the presence ($\bullet$) or absence (o) of Ca^{2+} and phosphatidylserine. In the latter measurements 100 μM EGTA was present. The difference between the two sets of experiments has also been plotted ($\blacktriangle$).

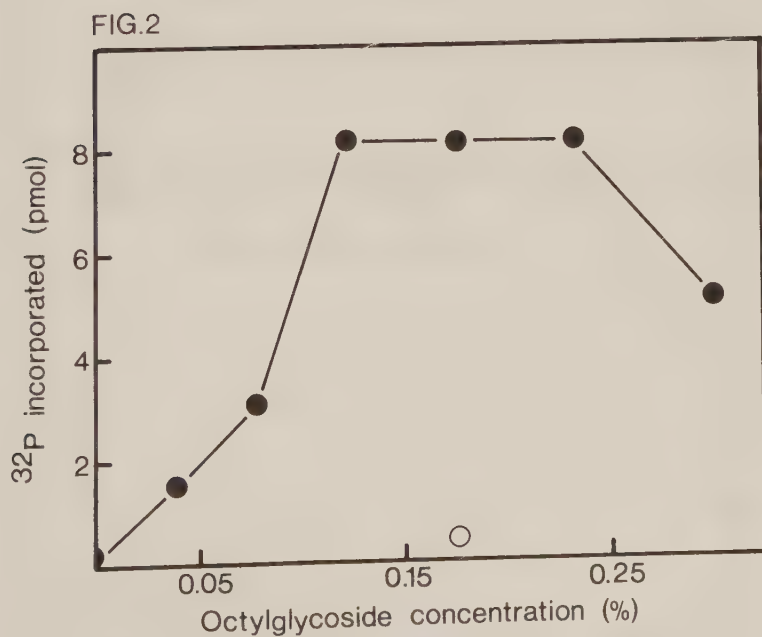
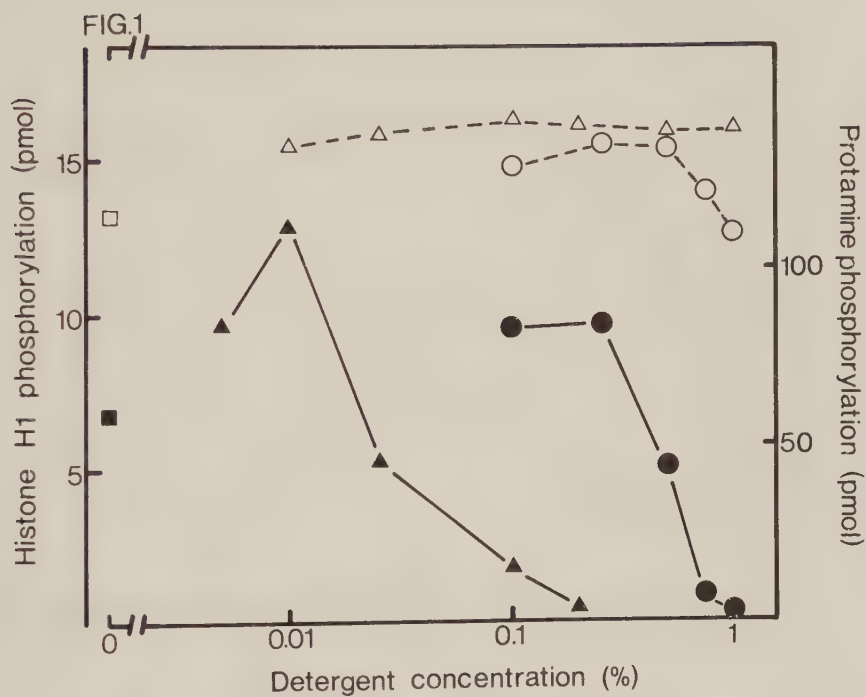


FIG.3

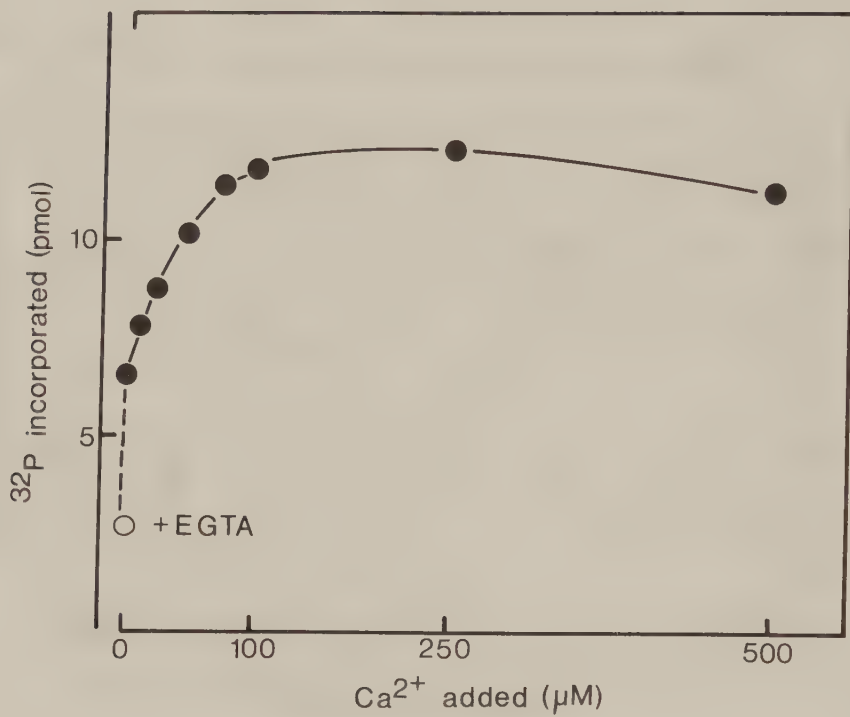


FIG.4

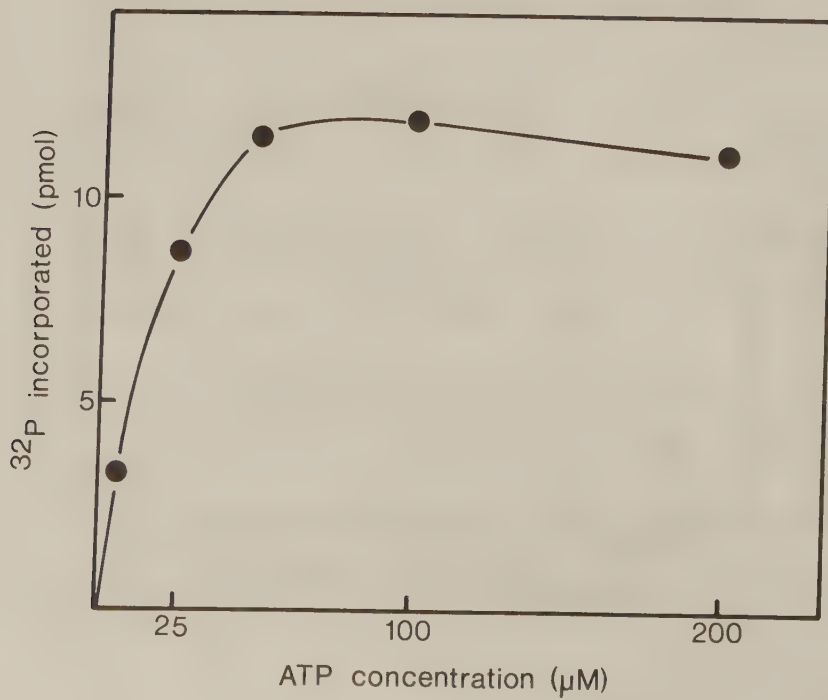
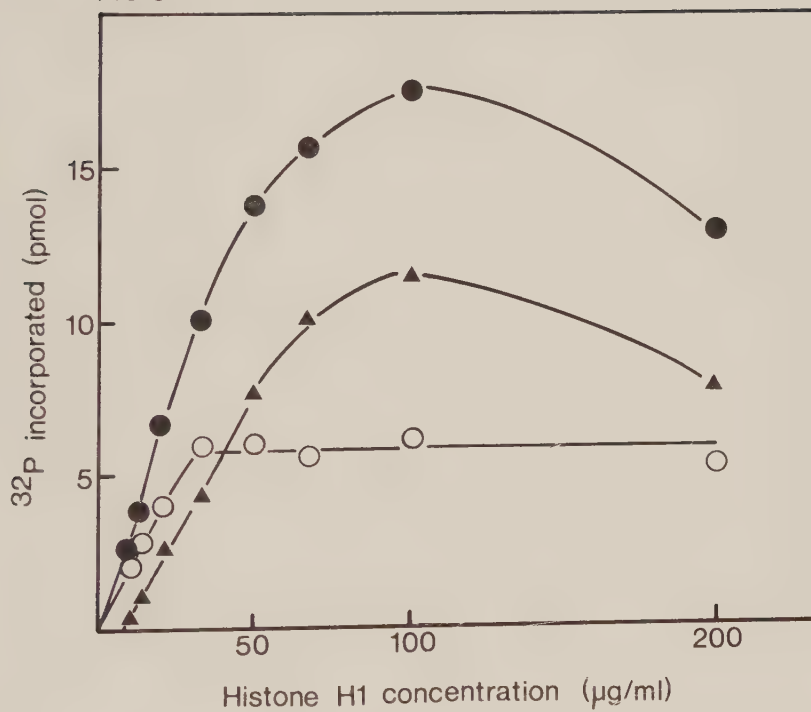


FIG. 5



MICROSOMAL Ca^{2+} - AND PHOSPHOLIPID-DEPENDENT PROTEIN KINASE -
IDENTIFICATION AND IN VITRO BINDING STUDIES

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A Ca^{2+} - and phospholipid-dependent protein kinase (CaPK) has been identified in rat liver microsomes. CaPK isolated from liver cytosol bound to smooth microsomes in the presence of 100 μM CaCl_2 . A saturation in binding was observed when a 5-fold excess of enzyme over that present in microsomes had become bound. The microsomal CaPK and 50% of the enzyme bound in vitro was not removed by EGTA treatment. This suggests that Ca^{2+} is required for the binding of CaPK to microsomes, but not for the retention of the enzyme on the membrane.

Key words: Ca^{2+} , liver microsomes, membrane binding, phospholipid,
protein kinase

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1. Introduction

The Ca^{2+} - and phospholipid-dependent protein kinase [1] (CaPK) is present in particulate and soluble fractions of various types of cells [2]. Takai et al. [3] showed that the enzyme was activated by membrane fractions in the presence of Ca^{2+} , and it was inferred, but not directly shown, that the enzyme would bind to membranes. In fact, later work suggests that CaPK bound to membranes cannot be detected unless the enzyme is solubilized first [2,4].

Recently we have devised a method for measuring CaPK in membrane fractions [4]. Approximately two thirds of the total enzyme activity in rat liver was found in particulate material, and more than 90% of the bound enzyme was present in microsomes [4]. It was not shown conclusively, however, that the bound enzyme was phospholipid-dependent, since phospholipids were present in the membrane extracts analyzed. We show in this communication that the microsomal enzyme is indeed CaPK. We have also examined some factors required for the enzyme to bind to microsomal membranes in vitro. Such model studies may be of interest in view of the rapid redistribution of CaPK between membranes and the soluble fraction detected recently [5], suggesting a strict control of CaPK-membrane interactions.

2. Experimental

2.1. Preparation of microsomes

Rat liver smooth microsomes were prepared as described [6] with the following modifications: the buffer used was 10 mM Tris-HCl, pH 7.4; MgCl_2 was omitted; 1 mM EDTA and 0.5 mM EGTA were present in the homogenization medium and the sucrose gradient. Smooth microsomes for DEAE-Sephacel chromatography were used immediately, whereas those to be used for binding studies were stored at -70° .

2.2. DEAE-Sephacel chromatography

Smooth microsomes were solubilized in 1% Triton X-100 and chromatographed on DEAE-Sephacel (Pharmacia) in the presence of 0.25% Triton X-100 [6].

2.3. Preparation of CaPK

CaPK was partially purified from the cytosol by DEAE-Sephacel chromatography [6] performed in the presence of 10^{-4} M cyclic AMP. The CaPK obtained was virtually free from cyclic AMP-dependent protein kinase as judged by its very slow phosphorylation of the peptide Leu-Arg-Arg-Ala-Ser-Val-Ala (provided by Dr. Ö. Zetterqvist, Uppsala). The specific activity with histone H1 as substrate was 1-2 nmol phosphate incorporated per min and mg protein in the presence of CaCl_2 and phosphatidylserine. The enzyme was stimulated more than 20-fold by CaCl_2 in the presence of phosphatidylserine.

2.4. Analyses

CaPK was analysed in the presence of octylglycoside [4]. When Triton was present (DEAE-Sephacel eluate) its concentration never exceeded 0.0125% in the analysis mixture, the concentration of phosphatidylserine was doubled and octylglycoside omitted. Protamine kinase was analysed as described [7]. Protein was determined [8] with bovine serum albumin as standard.

2.5. Binding studies

Smooth microsomes (approximately 0.5 mg of microsomal protein) were incubated at 0° with CaPK (approximately 0.15 mg) and CaCl_2 (or other additions) in a total volume of 250 μl . After 45 min 750 μl of 10 mM Tris-HCl, pH 7.4, was added and the samples were centrifuged at 105 000 g for 60 min. Pellets were suspended in 300 μl of the Tris buffer. The protease inhibitors leupeptin (2.5 $\mu\text{g}/\text{ml}$), pepstatin (1 $\mu\text{g}/\text{ml}$) and phenylmethylsulfonyl fluoride (350 $\mu\text{g}/\text{ml}$) were present in all solutions.

3. Results

3.1. Identification of CaPK in microsomes

A microsomal extract was chromatographed on DEAE-Sephacel, and the effluent was analyzed for protamine kinase and CaPK activities (Fig. 1). One peak of CaPK activity, coincident with the major protamine kinase peak, eluted at 0.14 M KCl. This enzyme was stimulated 8-10 times by CaCl_2 in the presence of phosphatidylserine using histone H1 as substrate, whereas it was virtually inactive in the absence of phospholipid. The specific activity was 4 nmol phosphate incorporated per mg protein. The recovery of CaPK on chromatography was quantitative, indicating that the Ca^{2+} -dependent activity of the extract represented CaPK rather than other Ca^{2+} -dependent protein kinases.

The two protamine kinase peaks eluting before and after CaPK constitute the catalytic subunit and the type II holoenzyme of cyclic AMP-dependent protein kinase, respectively 6 .

3.2. Binding of CaPK to microsomes

The binding of partially purified CaPK to smooth microsomes was tested at various concentrations of CaCl_2 (Fig. 2). Microsomes prepared in the presence of chelators to remove bound Ca^{2+} did not bind CaPK unless CaCl_2 was added. Maximum binding occurred at 100 μM CaCl_2 . Approximately 70% of the recovered kinase activity (in other experiments up to 90%) then sedimented with the microsomes. The same high degree of binding was obtained at 25 μM CaCl_2 when the volume of the incubation mixture for binding was increased 4-fold keeping the amount of microsomes constant (open circle). No binding of CaPK occurred at 500 μM MgCl_2 (square) unless CaCl_2 was present. Smooth microsomes prepared in the absence of chelators bound CaPK to a certain extent (20-30%) without addition of CaCl_2 .

Diolein enhances the stimulatory effect of phospholipids on CaPK activity at low Ca^{2+} concentrations [9] and has therefore been invoked in CaPK-membrane interactions [10]. Added diolein (40 $\mu\text{g/ml}$ incubation mixture) or intrinsic diacylglycerol generated in the membrane by hydrolysis of exposed phosphatidylinositol by a phospholipase C [11,12] did not affect the binding of CaPK to smooth microsomes at various Ca^{2+} concentrations. Thus, diolein does not seem to influence the binding of CaPK to microsomes.

3.3. Extent of CaPK binding to microsomes

When smooth microsomes were incubated with progressively more CaPK in the presence of CaCl_2 a maximum of around 5 times more enzyme bound than is usually found in the membrane [4]. There was no appreciable binding in the absence of CaCl_2 (open circles).

4. Discussion

Our results show that the Ca^{2+} -dependent protein kinase in microsomes also is phospholipid-dependent. CaPK has not been identified unequivocally in microsomes previously. In view of the abundance of the enzyme in microsomes [4], these are the major source of CaPK in rat liver cells.

We have earlier isolated and partly characterized both the microsomal and soluble CaPK as protamine-phosphorylating enzymes [6]. They had all properties examined in common, suggesting that CaPK does not undergo substantial changes when it becomes associated with membranes. The microsomal enzyme appeared located on the cytosolic side, and was released only by solubilization of the membrane.

The rapid translocation of CaPK from cytosol to plasma membranes in parietal yolk sac cells treated with phorbol esters [5] suggests that the binding of CaPK to membranes is under physiological control. However, nothing is known

about membrane factors involved in this binding. The activation of soluble CaPK by Ca^{2+} , phospholipids and diolein [1-3, 9] suggests that these factors may participate.

Our study shows that Ca^{2+} is required for binding in vitro, although this occurred at a much higher concentration of CaCl_2 than that present in the cytosol in vivo. However, the concentration of Ca^{2+} at the membrane surface in vivo may be rather high. The binding of CaPK to smooth microsomes prepared without chelators suggests that sites with high affinity for Ca^{2+} are involved. Removal of Ca^{2+} with EGTA does not cause the release of CaPK from microsomes [4]. Also about 50% of the enzyme bound in our studies was not released by EGTA. This indicates that the binding of CaPK to microsomes is Ca^{2+} -dependent, but that its retention in the membrane is Ca^{2+} -independent (pre-supposed Ca^{2+} was removed by EGTA).

If the binding in vitro occurs to specific sites (presumably involving proteins and/or phospholipids) it is expected to reach saturation at a level of CaPK not far exceeding that found in isolated membranes. The maximum amount of CaPK bound was 5 times that present in smooth microsomes. Since 50% of the bound CaPK was resistant to EGTA, a 2-3-fold excess of CaPK had become attached irreversibly. The binding of CaPK was not affected appreciably by prior treatment of microsomes with trypsin (10-20% decrease). Whether the CaPK attached irreversibly in vitro and the enzyme present in isolated membranes are bound in the same manner remains to be elucidated.

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Legends to Figures

Fig. 1. DEAE-Sephacel chromatography of a Triton X-100 extract of smooth microsomes. The phosphorylation of histone H1 was measured in the presence (●) and absence (○) of CaCl_2 and phosphatidylserine. ■, protamine phosphorylation. The specific activities of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ were 235 000 and 23 000 cpm per nmol in the histone H1 and protamine phosphorylations, respectively.

Fig. 2. The binding of CaPK to smooth microsomes at different concentrations of CaCl_2 . CaPK bound is expressed as % of recovered enzyme activity. The recovery of CaPK was around 140% up to 75 μM CaCl_2 , 100% at 100 μM CaCl_2 , and 50-60% above 100 μM CaCl_2 . ○, 25 μM CaCl_2 in a 4-fold increased volume of the enzyme-membrane binding mixture; □, 500 μM MgCl_2 instead of CaCl_2 .

Fig. 3. Maximum binding of CaPK to smooth microsomes. Various amounts of CaPK were added to smooth microsomes (0.3 mg membrane protein) in presence (●) or absence (○) of 0.5 mM CaCl_2 , and in a total volume of 1.0 ml. The mixture was incubated, centrifuged without further dilution, and analyzed for CaPK activity as described under Experimental. One unit is the amount of enzyme which catalyzes the incorporation of 1 nmol of phosphate into histone H1 per min at 22°.

FIG.1

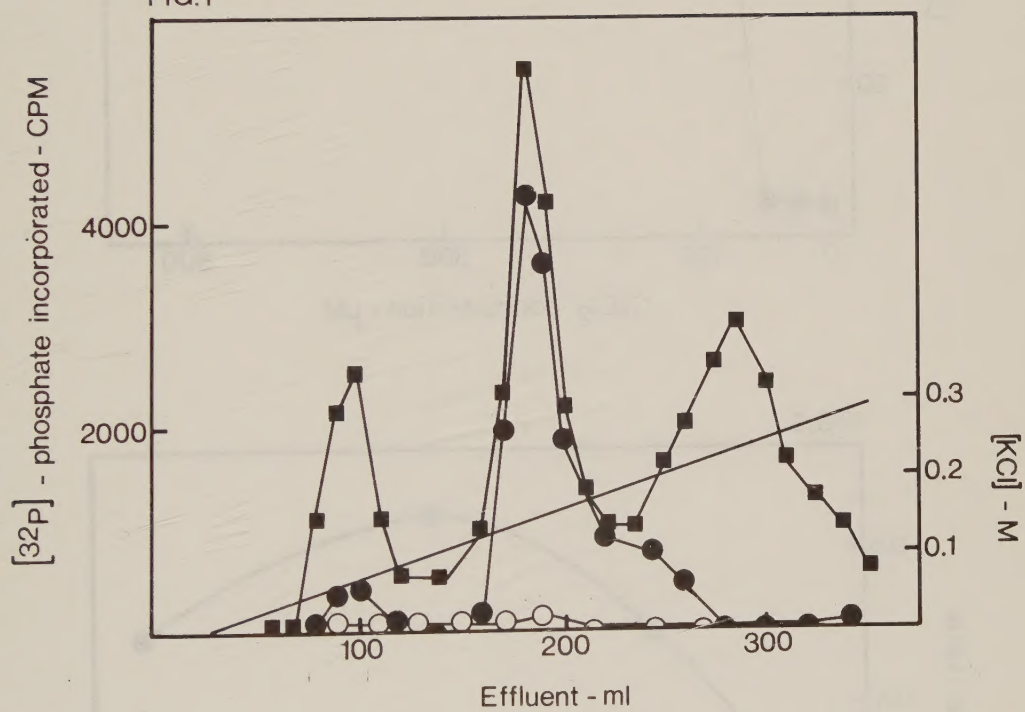


FIG.2

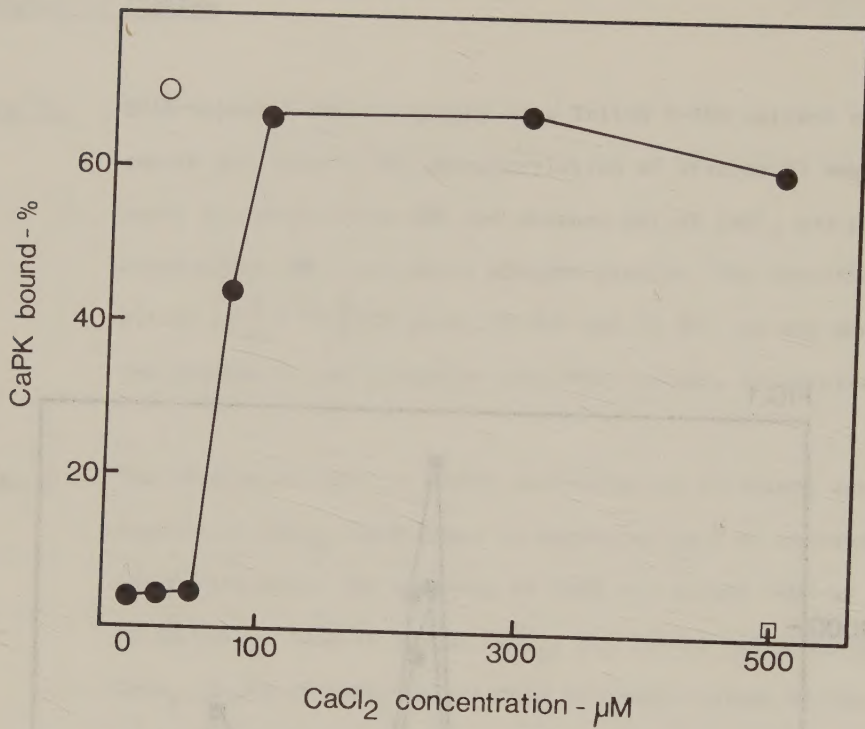


FIG.3

